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13. ABSTRACT (Maximum 200 Words)

The present program of research aimed at determining the neuroprotective activity of novel estrogens in both male and female animal models for brain ischemic damage. We proposed to achieve 4 technical aims over the course of 3 years. These aims related to a description of the activity of several estrogens against cerebral damage induced by middle cerebral artery occlusion in a rodent model, when the estrogens were administered prior to or following the ischemic event. The present grant was immensely successful in achieving its technical objectives and in its productivity. During the 3.5 years of this grant, we published 27 full length technical papers, have 7 papers *in press*, have submitted an additional 14 manuscripts and have 5 manuscripts *in preparation* based upon the results of the studies proposed. Additionally, the research completed in this grant period has been described in 41 published abstracts. Collectively, these studies provided the knowledge that estrogen therapy and androgen reduction therapy are useful in protection of the brain tissue from damage related to the activities of U.S. Army male and female personnel.

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(4) Introduction: The present grant has been immensely success in achieving its technical objectives and in its productivity. During the 3.5 years of this grant, we published 27 full length technical papers, have 7 papers *in press*, have submitted an additional 14 manuscripts and have 5 manuscripts *in preparation* based upon the results of the studies proposed. Additionally, the research completed in this grant period has been described in 41 published abstracts. The present program of research aims at determining the neuroprotective activity of novel estrogens in both male and female animal models for brain ischemic damage. We have proposed to achieve 4 technical aims over the course of 3 years and requested a one year no cost extension, due to my move from the University of Florida to the University of North Texas Health Science Center at Fort Worth (UNTHSCFW). These aims relate to a description of the activity of several estrogens against cerebral damage related to middle cerebral artery occlusion in a rodent model, when the estrogens are administered prior to or following the ischemic event. Also, we assessed the potential mechanism(s) of the neuroprotective activity of estrogens in these models by determining the extent to which estrogens reduce oxidative damage associated with stroke and then assess the involvement of signal transduction processes and anti-apoptotic proteins in the neuroprotective activity of estrogens. Collectively, these studies have provided the knowledge needed to demonstrate that estrogen therapy would be useful in protection of the brain tissue (and other tissues) from damage related to the activities of U.S. Army male and female personnel.

(5) Body:

Background: This report covers the period of August 1, 1999 to May 31, 2003 for research funded by the grant from the USAMRMC numbered DAMD 17-99-1-9473. In the Fall of 2001 and again in the summer of 2002, Major Chessley Atchison, Ph.D., conducted a site-visit at the UNTHSCFW. The progress reported in this document has also been reported to Dr. Atchison.

We have achieved and surpassed all of the technical aims of the grant. This progress is reported below.

Technical Aim 1: To assess the prophylactic and post-treatment neuroprotective effects of novel estratrienes in an animal model of cerebral ischemia.

Although not specifically funded by this grant, we have a program of drug synthesis that provided new compounds to our stroke and brain trauma program. During the course of this grant, we synthesized and completed *in vitro* neuroprotective evaluations of more than ***60 novel estrogens*** to identify candidate compounds for activity in stroke models.

The prophylactic neuroprotective effects of novel estratrienes in an animal model of cerebral ischemia

To date, we have tested in our by middle cerebral artery (MCA) occlusion model, 8 of these compounds and subjected these data to peer review. The compounds tested in our MCA occlusion model are the following. The potent estrogens, 17 β-estradiol and

estrone; the weak estrogen, 17α-estradiol; the complete enantiomer of 17 β-estradiol that does not have estrogen agonist activity, Ent-estradiol, a brain-enhanced chemical delivery system for estradiol, E2-CDS; the non-feminizing estrogen, 2-adamantyl-estrone (Zyc-3); the non-feminizing estrogen enantiomer of 17-desoxyestradiol (Zyc-13); and a novel prodrug form of estrogen, estrone-10 hydroxy-quinol. Each of these estrogens was as effective as the potent estrogen, 17 β-estradiol, in protecting brain tissue from the damage caused by MCA occlusion.

We have previously reported that 17 β-E2 is a potent neuroprotectant when administered prior to the onset of MCA occlusion. Initially, we demonstrated that administration of 17 β-E2 by either a sc or an iv formulation at 1 day prior to MCA occlusion protected animals from much of the cerebral damage related to the infarct (Simpkins et al., 1997). Subsequently, we demonstrated that a slow release preparation of 17 β-E2, administered beginning 1 week prior to the occlusion was potently effective in protecting brain tissue from ischemia damage (Simpkins et al., Unpublished Observations). Similarly, administration of 17 β-E2 at 2 hours prior to the onset of the infarct was protective (Zhang et al., 1998). Finally, using nuclear magnetic resonance (NMR) imaging to perform real time imaging of the development of ischemic damage, we demonstrated that administration of 17 β-E2 at 2 hours prior to the onset of the occlusion can essentially protect much of the brain tissue from the damaging effects of occlusion (**Appendix A**).

Novel estrogens were also assessed for prophylactic neuroprotection. We completed an assessment of the protection against cerebral damage related to MCA occlusion is animals pre-treated with 17 α-estradiol (17 α-E2), through a subcutaneous

Silastic® tube containing the steroid at two different doses. We observed that 17 α-E2 provided protection of both cortical and subcortical brain areas from damage induced by the occlusion. The extent of the protection provided by 17 α-E2 was similar to that afforded by 17 β-E2.

We tested the complete enantiomer of 17 β-E2, Ent-E2, for its neuroprotective activity in an MCA occlusion model of brain damage. We observed that ent-E2 was as effective as 17 β-E2 in protecting both cortical and subcortical tissue from damage **(Appendix B)**.

Additionally, two of our newly synthesized compounds, ZYC-3 and ZYC-13 has completed *in vivo* assessments in our MCA occlusion model. We have observed that both ZYC-3 and ZYC-13 are potently neuroprotective against ischemia induced brain damage **(Appendix C)**. Collectively, these data indicate that non-feminizing estrogens, like 17 α-E2, Ent-E2, ZYC-3 and ZYC-13 are potent neuroprotectant in at least one model for cerebral damage, the MCA occlusion model for stroke.

Recently, we have assessed a novel prodrug of estrone for its neuroprotective activity on MCA occlusion. We determined that estrone-10 hydroxy-quinol was an oxidative metabolite of estrone, that could be reduced to the active estrogen, estrone, in the brain. We administered estrone-10 hydroxy-quinol prior to MCAO and demonstrated that it was an protective as estrone in this model. As estrone-10 hydroxy-quinol is believed to be inactive as an estrogen, this prodrug of estrone, could be a useful means of delivery of neuroprotectants to the brain.

In summary, while we proposed to test three compounds during the course of this grant period, we completed testing on 8 compounds and showed all to be efficacious.

These data provide support for the use of these non-feminizing estrogens in the protection of brain tissue from the effects of stroke as well as brain trauma in male and female military personnel.

Post-treatment efficacy of estratrienes

We have completed and published three papers assessing the efficacy of estrogens in protecting the brain from damage MCA occlusion. First, we demonstrated that a low dose of 17β-estradiol (100 μg/kg) could protect from the damaging effects of MCA occlusion for as long as 3 h after the onset of the ischemia (**Appendix D**). Subsequently, we assessed 17β-estradiol at higher doses (500 μg/kg and 1000μg/kg) at a longer post-ischemia interval (6 hours) and determined that animals were also potently protected (**Appendices E & F**). These data indicate that estrogens can be administered for as long as 6 h after an ischemic event and still protect the brain from injury. Implicit in these data is the potential for use of estrogen compounds to protect brain tissue in military operation after an ischemic or traumatic event has occurred. This post-trauma efficacy of estrogens has potential use in the treatment of brain injured soldiers.

Extension of these observation to other stroke models and other tissues

We used the observations that estrogens are potent neuroprotectants in the brain against MCA occlusion to extend these observations to other models as well as other tissues. First, we demonstrated that estrogens protect brain tissue form damage caused by subarachnoid hemorrhage (SAH). We completed and have now published (**Appendix G**) an assessment of the prophylactic protective effects of 17β-estradiol in a model for SAH

that we developed. Animals were administered 17β-estradiol at a dose of 100 μg/kg at 2 hours prior to the induction of an intraluminal hemorrhage using a thread that punctured the anterior cerebral artery. Pre-treatment with 17β-estradiol preserved blood flow during the bleeding episode, but did not effect the bleeding volume, and markedly reduced the extent of cerebral ischemic damage related to the bleeding episode (**Appendix G**). These data indicate that treatment with 17β-estradiol can provide protection from bleeding as well as occlusion type strokes.

Further, we assessed the effects of 17β-estradiol on hippocampal protection from global ischemia. The hippocampus is one of the most vulnerable areas of the brain to ischemia. In our model, we produced ischemia in female rats by a four-vessel occlusion. We demonstrated that pre-treatment with 17β-estradiol prevented most of the damage to the hippocampus caused by the ischemic event (**Appendix H**).

Finally, we extended this observation that estrogens protect brain tissue from ischemia, to the skeletal muscle, heart, retina and lens of the eye (**Appendices I, J**). In all of these tissues, estrogen pretreatment was very protective. These data suggest that estrogens can be used by military personnel to protect a variety of tissues form ischemia and trauma.

Male Subjects

We assessed the possibility that estrogen could provide cerebral protection in male subjects as well as in females. In an initial study, published prior to the onset of this grant (Hawk et al, 1997) we reported that treatment for 1 week with 17β-estradiol by a sc

Silastic® tube containing the steroid, resulted in protection from brain damage induced by MCA occlusion equivalent to that observed in females. Interestingly, however, this protection form brain damage was also observed when males were castrated. This raised the possibility that the effects of estrogens in males were related to their capacity to reduced serum androgens, such as testosterone. To test this possibility, we administered estrogens, but maintained serum testosterone levels relatively constant through sc implants of Silastic® tubes containing the male sex steroid. When testosterone concentrations were maintained, estrogens lost most of their ability to protect brain tissue. These data indicated that serum androgen reduction was a key event in the neuroprotection afforded by estrogens.

In view of this surprising finding, we conducted a series of studies to determine the effects of androgen reduction, through a variety of means, on brain damage subsequent to an infarct. First, we administered the potent LHRH agonist, luprolide, which nearly completely suppresses secretion of LH and hence of testosterone, twice daily for one week. Luprolide reduced testosterone concentration to nearly undetectable levels after a week of administration. These animals were subjected to MCA occlusion and 24 hours later lesion size was evaluated. Luprolide reduced lesion size by 50%, the same extent of protection seen with estrogen pretreatment (Cartright et al., 2000).

Finasteride is a 5α-reductase inhibitor that prevents the conversion of testosterone to 5 α-dihydrotestosterone (HDT). We administered finasteride to male rats, at 6 hours prior to MCA occlusion and evaluated the size of the ischemic infarct 24 hours later. A single dose of finasteride reduced infarct size in male rats by about 40% (Cartright et al., 2000).

To determine if a non-pharmacological means of reducing testosterone would also protect brain from ischemic damage, we subjected male rats to the stress of administration of an anesthetic (or control animals were left undisturbed) at 6 hours prior to the MCA occlusion. Animals that were subjected to stress showed a major reduction in serum testosterone concentrations and a 50% reduction in infarct size (**Appendix K & L**).

Finally, to fully characterize the duration of testosterone reduction needed to protect animals from ischemic damage, we created a "testosterone clamp" by castrating male rats and implanting them with 2 testosterone filled Silastic® tube. These implants produced serum testosterone concentration which were equivalent to that seen in intact rats (**Appendix K & L**). Removal of these implants caused an 85% reduction in testosterone concentrations within 1 hour and undetectable levels of the hormone within 2 hours (**Appendix K & L**). MCA occlusion was initiated at 0 (no removal of the pellets), 1, 2, 4, or 6 hours after removal of the testosterone tubes. We observed a time-dependent reduction in the ischemic damage, with a 50% reduction seen at 6 hours (**Appendix K & L**). These data indicate that the removal of testosterone for as little as 6 hours can substantially protect the male brain from the damaging effects of ischemia.

To determine if androgens are directly toxic to neurons, we use an *in vitro* tissue culture model of glutamate toxicity in HT-22 mouse hippocampal cells. While estrogen treatment potently protected cells from this insults, testosterone exposure potentiated the glutamate toxicity, suggesting that androgens are directly toxic to neurons (**Appendix K & L**).

Technical Aim 2: To determine the effects of novel estratrienes on oxidative damage during and following a cerebral ischemic event.

To serve as an initial screen for the efficacy of estratrienes on oxidative damage, we assessed the effects of 10 selected compounds on a marker of lipid peroxidation in brain extracts of rats. We demonstrated that the potency of these compounds in protecting from lipid peroxidation correlated well with their potency in protecting neurons *in vitro*, from neurotoxicity. These data indicate that an anti-oxidant activity of estratrienes is a likely contributor to their neuroprotective activity.

We investigated the antioxidant activity of estrogens further by assessing the ability of estrogens to undergo oxidation-reduction cycling. We discovered that estrogens are rapidly and readily metabolized to a 10-hydroxy-quinol metabolite that can and is reduced back to its parent estrogen. This discovery allowed us to synthesize a variety of compounds based upon the 10-hydrox-quinols and demonstrate their neuroprotective activity. These data suggest that the potent anti-oxidant activity of estrogens are due to their ability to enter lipid membranes and there undergo redox cycling and thereby prevent oxidation of vital membrane lipids.

An indicator of cellular oxidation is production of NFκB. Normally, NFκB is maintained in an inactive state through the coupling to IκB. Reactive oxygen species (ROS) cause phosphorylation of IκB, which then dissociates from NF-κB, allowing it to translocate to the nucleus and there activate a variety of potentially harmful genes. We conducted studies to determine the effects of MCA occlusion on the production of both

NFκB (active form) and phosphorylated IκB (inactive form). We observed a marked increase in both NFκB and the phosphorylated form of IκB in OVX rats subjected to MCA occlusion (**Appendix M**). The activation of NFκB was an early event, occurring as early as 2 hours after the onset of reperfusion following MCAO. Additionally, the activation of NFκB occurred in neurons that were undergoing apoptosis, as evident by the colocalization of NFκB with TUNEL staining (**Appendix M**). Finally, the activation of NFκB, as well as the induction of apoptosis was largely prevented by estrogen treatment prior to the onset of the MCAO (**Appendix M**). These data indicate that ischemia/reperfusion is a strongly pro-oxidant event and that estrogens are effective in preventing this oxidative event.

Technical Aim 3: To determine the effects of novel estratrienes on CREB expression and phosphorylation following a cerebral ischemic event.

We conducted studies to determine the effects of MCA occlusion and reperfusion on levels of CREB and phosphorylated CREB (P-CREB). We observed that MCA occlusion and reperfusion decreased levels of both CREB (expression of the protein) and P-CREB (activation of the protein) in the infarcted brain regions. Pretreatment with 17β-estradiol prevented these changes in both CREB and P-CREB. These data indicate that CREB expression and CREB phosphorylation are responsive to the cerebral ischemia and that estrogens prevent this response. As CREB phosphorylation may be a neuroprotective signal, the ability of estrogens to prevent this response to ischemia may indicate a neuroprotective mechanism. In view of our *in vitro* data indicating that both feminizing

as well as non-feminizing estrogen induced CREB phosphorylation, we fully expect that other non-feminizing estrogens will activate CREB as a mechanism of neuroprotection.

Inasmuch as ischemic events, such as occurs with stroke and trauma, are strongly pro-oxidant (See above), we expanded this aim by assessing the effects of MCA occlusion on a novel pathway for the formation of neurofibillary tangles in neurons. This approach was taken because pro-oxidant stress leads to signaling that cases neurons to attempt a catastrophic mitosis, and in the process activated apoptosis (Programmed cell death) as well as tau hyperphosphorylation, a required event in the formation of neurofibillary tangles in neurons. We observed and have submitted a manuscript (**Appendix N**) that describe that MCA occlusion rapidly enhances the activity of a variety of proteins known as cyclin-dependent kinases (required for cell division to occur). These enzymes start neurons into cell division (catastrophic mitosis) that leads to apoptosis. In the process, these same enzymes cause tau hyperphosphorylation and neurofibillary tangles in neurons in surviving neurons. We have also have observed that a cyclin-dependent kinase called cdk-5, was particularly response to oxidative stress and was an initial step in tau hyperphosphorylation. This discovery may serve to explain the observations that those individuals who survive a stroke are 3-12 times more likely to progress to dementia than control subjects. Finally, we have observed that estrogen treatment prevents this cascade of events following MCA occlusion.

Technical Aim 4: To determine the effects of novel estratrienes on BCL-2 expression following a cerebral ischemic event.

We have demonstrated that MCA occlusion decreases BCL-2 concentrations in affected areas of the brain and that 17β-estradiol prevents this BCL-2 response. These data were not unexpected inasmuch as estrogens have been shown by other to preserve BCL-2 concentrations in the face of pro-oxidant stress *in vitro* and in MCA occlusion models.

In as much as BCL-2 appears to serve to protect the mitochondria from pro-oxidant insults, we investigated the effects of both 17β-estradiol and non-feminizing estrogens on mitochondrial function. Our functional assessment of the mitochondria included production of ATP, mitochondrial membrane potential collapse (a neurotoxic event) and the required Ca^{2+} influx into the cytoplasm and then into the mitochondria. Two different pro-oxidant stresses resulted in a depletion of ATP, accumulation of cytoplasm and mitochondrial Ca^{2+} ; collapse of mitochondrial membrane potential and subsequently, cell death (**Appendices J, P, Q**). Pretreatment with 17β-estradiol largely prevented these effects of pro-oxidants in neurons (**Appendices P, Q**) as well as lens cells (**Appendix J**). Additionally, the non-feminizing estrogens, 17 α-estradiol and Ent-E2 were as effective in protecting mitochondrial functions as was 17β-estradiol in both neurons (**Appendices P, Q**) and lens cells (**Appendix J**). These data strongly indicate that a primary mechanism of estrogen neuroprotection in stroke and trauma is through the protection of mitochondria.

(6) Key Research Accomplishments:

- Two feminizing estrogens and 6 non-feminizing estrogens like were shown to be potent neuroprotectants against cerebral damage induced by brain ischemia.

- This neuroprotectant activity can be achieved even when the estrogen is administered for up to 6 h after the ischemic event.
- The protective effects of estradiol can be observed in male subjects, and appears to have a strong component related to estrogen-induced androgen reduction.
- Acute androgen reduction is potently neuroprotective in male subjects.
- Transient cerebral ischemia causes an activation of oxidation and oxidative signaling and these effects are largely preventable by estrogens.
- Brain levels of both CREB and phosphorylated CREB are decreased following cerebral ischemia/reperfusion and estrogens prevent these effects.
- BCL-2 levels following MCA occlusion are reduced and the estrogens prevent these effects.
- Cerebral ischemia causes an activation of cyclin-dependent kinases leading to apoptosis, catastrophic cell division and hyperphosphorylation of tau. Estrogens ameliorate all of these effects.
- Pro-oxidant events, such as those induced by cerebral ischemia, lead to a precipitous decline in mitochondrial function and neuronal death. Estrogens protect mitochondrial function and this is a primary mechanism of their neuroprotection.

(7) Reportable Outcomes:

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1. Watson, D. G. and **J.W. Simpkins**, Estradiol exposure alters the activity and intracellular distribution of PKC epsilon in an immortalized hippocampal cell line, Neuropharmacology, submitted 2003.

2. Watson, D. G. and **J.W. Simpkins**, Protein kinase C inhibition or down-regulation mimics and enhances estradiol-induced neuroprotection in an immortalized hippocampal cell line, Neuropharmacology, submitted 2003.

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(8) Conclusions: The present grant has been immensely success in achieving its technical objectives and in its productivity. During the 3.5 years of this grant, we published 27 full length technical papers, have 7 papers in press, have submitted an additional 14 manuscripts and have 5 manuscripts in preparation based upon the results of the studies proposed. Additionally, the research completed in this grant period has been described in

41 published abstracts. This enormous effort resulted in our achieving all of the technical aims of the grant.

As a result of these studies, we believe that we have sufficient preclinical data with which to initiate a clinical trial (from other sources of funds) to assess the efficacy of estrogens in protection of the brain from damage due to cerebral ischemia during stroke or brain trauma.

(9) References:

The references are listed as Reportable Outcomes of the grant.

(10) **Appendices:** Appended materials are identified in the text (Reportable Outcomes Section) and are attached. Due to large volume of published materials, only the most identified publications are attached as appendices. The attached appendices are as follows:

Appendix A: Shi, J., J.D. Bui, S.H. Yang, T.H. Lucas, D. L. Buckley. S.P. Blackband, M.A. King, A.L. Day and **J.W. Simpkins**, Estrogens decrease reperfusion-associated cortical ischemic damage: a MRI analysis in a transient focal ischemia model. *Stroke* 32: 987-992, 2001.

Appendix B: Green, P. S., S.-H. Yang, K. R. Nilsson, A. S. Kumar, D. F. Covey, **and J. W. Simpkins** The Nonfeminizing Enantiomer of 17ß-Estradiol Exerts

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Appendix I: Persky, A. M., Persky, A.M., P.S. Green, L.A. Stubley, C.S. Howell, L. Zaulyanov, G.A. Brazeau and **J.W. Simpkins**, Protective effect of estrogens against oxidative damage to heart and skeletal muscle in vivo and in vitro. Proc Soc Expt Biol Med, 223: 59-66, 2000.

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Appendix A

Estrogens Decrease Reperfusion-Associated Cortical Ischemic Damage

An MRI Analysis in a Transient Focal Ischemia Model

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Background and Purpose—Early identification of irreversible cerebral ischemia is critical in defining strategies that influence neuronal survival after stroke. We used MRI to investigate the effects of 17β-estradiol (E2) on the temporal evolution of focal ischemia.

Methods—Female rats were ovariectomized and divided into 1 of 2 groups: ovariectomy alone (OVX; n=4) or ovariectomy with estrogen replacement (OVX+E2; n=3). Both groups were then subjected to 1-hour middle cerebral artery occlusion (MCAO), with the use of a standardized endovascular monofilament model, followed by reperfusion. Sequential diffusion-weighted (DWI) and T2-weighted (T2WI) MRI were obtained during and after the MCAO. In separate groups of animals (n=5 for OVX and OVX+E2), cerebral blood flow (CBF) was measured by laser-Doppler methods before, during, and after occlusion.

Results—DWI detected similar lesion characteristics during MCAO in both groups. In the OVX group, lesion size did not change during reperfusion, but the signal intensity ratio increased early and stabilized during the latter stages. In contrast, DWI lesion size decreased during reperfusion in OVX+E2 rats by 50% to 60% ($P<0.05$), a size reduction almost exclusively limited to cortical regions. During MCAO, the signal intensity ratio in OVX+E2 rats was reduced compared with OVX rats. Reperfusion further attenuated the signal intensity ratio in cortical but not subcortical regions ($P<0.05$ versus OVX). T2WI revealed no lesions in either group during MCAO, but it detected lesion sizes similar to that of DWI during reperfusion. Furthermore, similar patterns and magnitudes of estrogen treatment–related decrease in lesion size were noted after reperfusion. T2WI demonstrated less intense signal intensity ratio changes in both groups compared with DWI. There were no differences in CBF between groups either during occlusion, early reperfusion, or 1 day after reperfusion.

Conclusions—This study strongly suggests that estrogens selectively protect cortical tissue from ischemic damage during MCAO and that this protection is exerted during both the occlusion and reperfusion phases of ischemia and does not involve an estrogen-related change in CBF. (***Stroke***. **2001;32:987-992.**)

Key Words: cerebral ischemia, focal ■ estrogens ■ magnetic resonance imaging ■ neuroprotection ■ reperfusion injury

Stroke ranks as the third leading cause of death and the leading cause of disability in the United States.[1] Stroke patients must not only survive the acute stages of infarction but must then cope with significant mental, physical, and economic stresses associated with neurological impairment. When one considers the cost in both loss of life and loss of self-esteem and productivity, the need for effective therapeutic interventions is obvious. Most strokes occur when perfusion to the middle cerebral artery (MCA) is reduced by a clot within the major cerebral arteries, producing a region of focal cerebral ischemia and a subsequent cascade of neuronal and microvascular changes ultimately leading to infarction.[2] Experimental ultrastructural evidence suggests that some of the damage occurs in the interval of reduced or absent perfusion (the occlusion phase), but most arises during the reperfusion stage, after flow has been restored by clot lysis or opening of collateral channels.[3] Since new thrombolytic therapy can now dissolve clots and restore arterial patency, the search for neuroprotective agents that can blunt the reperfusion-

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Stroke is available at http://www.strokeaha.org

TABLE 1. Physiological Parameters in Rats Subjected to Transient MCAO (n=4 for Each Group)

Group	MABP, mm Hg	pH	Pco_2	Po_2	Hematocrit, %
Before MCAO					
OVX	101±8	7.29±0.03	55±3	67±4	42±1
OVX+E2	90±3	7.27±0.02	61±2	69±4	43±2
Ischemia 30 min					
OVX	124±4	7.33±0.02	50±4	70±13	41±1
OVX+E2	116±7	7.31±0.05	51±5	70±8	43±3
Reperfusion 30 min					
OVX	99±11	7.35±0.01	45±1	74±5	44±1
OVX+E2	92±10	7.28±0.06	56±7	76±9	43±3

associated injury and elongate the tissue interval for safe intervention assumes critical strategic importance.[4]

Observations from our and other laboratories indicate that estrogens are potent neuroprotective agents and decrease focal and global ischemia-induced lesion size by as much as 50%.[5–15] An understanding of the events affected by estrogen during occlusion and reperfusion will allow us to define the therapeutic window for application of estrogens in stroke. The histological methods used in these previous studies limited our ability to dynamically assess the protective effects of estrogen during occlusion and reperfusion.

MRI can provide a wealth of critical information about the initiation, progression, and localization of cerebral ischemic events during their occurrence. Diffusion-weighted MRI (DWI), a sensitive indicator of random movement of water molecules, is thought to reveal the early changes associated with stroke-induced cytotoxic edema.[16] On the other hand, conventional T2-weighted MRI (T2WI), a sensitive indicator of vasogenic edema that occurs later in the pathophysiology of stroke, can detect subacute ischemic damages, even though it fails to show acute ischemic changes.[16] As such, MRI can quantify the progression of 2 major pathological consequences of cerebral infarction. In the present study we applied MRI techniques to noninvasively analyze, for the first time, the temporal and spatial effects of 17β-estradiol (E2) in focal cerebral ischemic events.

Materials and Methods

Animals

Sprague-Dawley female rats (250 g body wt) purchased from Charles Rivers Laboratories, Inc (Wilmington, Mass), were housed in pairs in hanging, stainless steel cages in a temperature-controlled room (25±1°C) with daily light cycle (light on 7 AM to 7 PM daily) for a minimum of 3 days before surgery. All rats had free access to Purina Rat Chow and tap water. All procedures performed on animals were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Florida before initiation of the study. Two weeks before the focal ischemia was induced, all rats were ovariectomized to eliminate endogenous estrogens. Rats in the ovariectomy with estrogen replacement (OVX+E2) group were administrated a single dose of E2 (100 μg/kg) 2 hours before focal ischemia surgery, while those in the ovariectomy alone (OVX) group received an oil vehicle injection. The sample size needed for the MRI study was calculated on the basis of published observations by us and others that E2 treatment caused a 50% reduction in MCA occlusion (MCAO)–induced lesion size.[5–15] A small number of animals sufficient to show statistical significance were assigned to each group.

Focal Ischemic Model

MCAO was achieved according to the methods described previously.[5] Briefly, after administration of anesthetics, the left common carotid artery, external carotid artery, and internal carotid artery on the left side were exposed and dissected through a midline cervical incision. A 3-0 monofilament suture was introduced into the left internal carotid artery lumen and gently advanced until resistance was felt, indicating MCAO and compromised blood flow. The suture

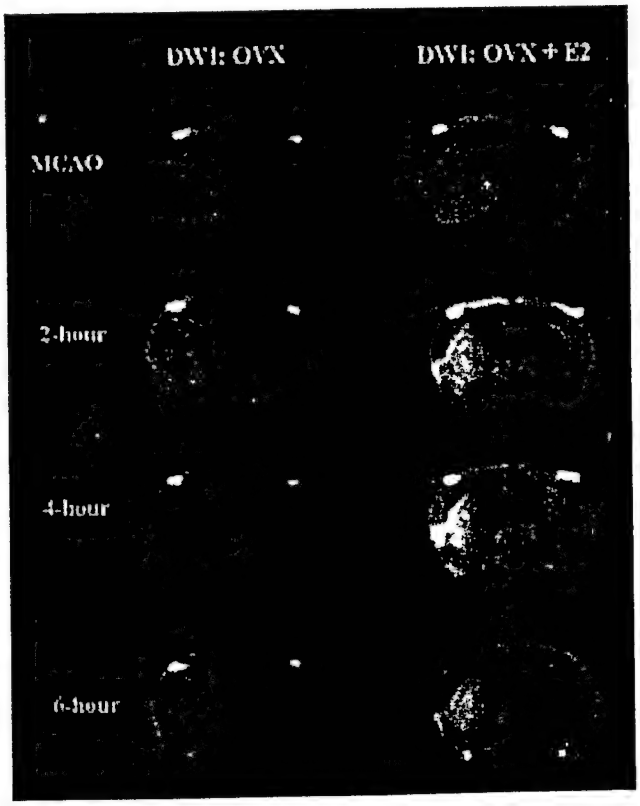

Figure 1. Sequential DWI from representative OVX and OVX+E2 rats during MCAO and after reperfusion. Two weeks after ovariectomy, female rats were divided into OVX (n=4) and OVX+E2 (n=3) groups. Both groups were then subjected to 1-hour MCAO. Sequential DWI were then obtained for each animal beginning at 30 minutes of the MCAO and at 2, 4, and 6 hours after monofilament removal (reperfusion interval). The imaging sections shown were captured at 9 mm caudal to the end of olfactory bulb.

was kept in place for 60 minutes and then withdrawn to allow MCA reperfusion. The operating procedure was performed within 20 minutes with little bleeding. Rectal temperature was monitored and maintained between 36.5°C and 37.0°C during the entire stroke procedure.

Magnetic Resonance Imaging

Imaging was performed in a 4.7-T, 33-cm magnet with a Brucket console with an actively shielded gradient set capable of 220 mT/m. The animals were supported on a cradle, and their heads were placed in a home-built birdcage coil with a 5-cm outer diameter (operating in quadrature transmit/receive mode). After the acquisition of scout images, 6 coronal plane images were prescribed beginning 3 mm behind the olfactory bulb. The slices were each 1.5 mm thick and were separated by 2 mm. All images were acquired over a 5-cm field of view with a 128×128 matrix (0.39×0.39-mm in-plane resolution), with a repetition time of 1.75 seconds and 2 signals averaged. Each set of 6 images was acquired in 7.5 minutes. DWI was performed with a standard pulsed gradient, spin-echo technique with an echo time of 33 ms. The gradient pulses were each applied for 9 ms and were separated by 13 ms around the 180° refocusing pulse. The gradient amplitude used was 152 mT/m, resulting in a b value of 1400 s/mm^2. T2WI was performed with a standard spin-echo technique with an echo time of 75 ms. Both DWI and T2WI were captured sequentially for each animal (n=4 for OVX and n=3 for OVX+E2 group) at 30 minutes during MCAO (the occlusion interval) and 2, 4, and 6 hours after withdrawal of the monofilament (the reperfusion period).

Quantification of Ischemic Lesion Sizes and Intensity

The ischemic lesion sizes and lesion intensity of the MR images were anatomically measured with Image-Pro Plus software (Media Cybernetics). The lesion area was subdivided into cortical and subcortical areas according to neuroanatomic landmarks. The percentage of the lesion size over the whole brain coronal section was calculated. The lesion intensity ratio was calculated, with the intensity of the nonlesioned hemisphere assigned a value of 1.

Cerebral Blood Flow Measurement

Cerebral blood flow (CBF) was measured in a separate group of rats (n=5 in each group for OVX and OVX+E2) that underwent focal ischemia surgery by methods that we have previously described in detail.[8] A middle line section exposed the small area around bregma. Two symmetrical holes were drilled through the skull and adjacent to the dura. These 2 holes were located at 1.5 mm posterior to and 3.5 mm left/right of bregma. Two probes of a digital laser perfusion monitor (MICROFLO DSP, Oxford Optronix Ltd) were placed on the dura to record the second-to-second change in CBF before, during, and after MCAO. Recordings were made from each animal for at least 10 minutes before occlusion, during the entire hour of occlusion, and beginning at 10 minutes of reperfusion and again at 24 hours after the occlusion. For each recording period, data representing a 10-minute period of stable CBF measurements were used to determine CBF for that sample period for each animal.

Physiological Parameters

Physiological parameters were monitored in a separate group. The left femoral artery was catheterized for mean arterial blood pressure monitoring and arterial blood sampling in the OVX and OVX+E2 animals (n=4 in each group). Physiology parameters were measured with a portable clinical analyzer (i-STAT).

Statistical Analysis

The Mann-Whitney U test was applied to determine the significance of the difference between OVX and OVX+E2 groups. R^2 was calculated to analyze the coherence of the 2 measurements. A value of $P<0.05$ was considered significant.

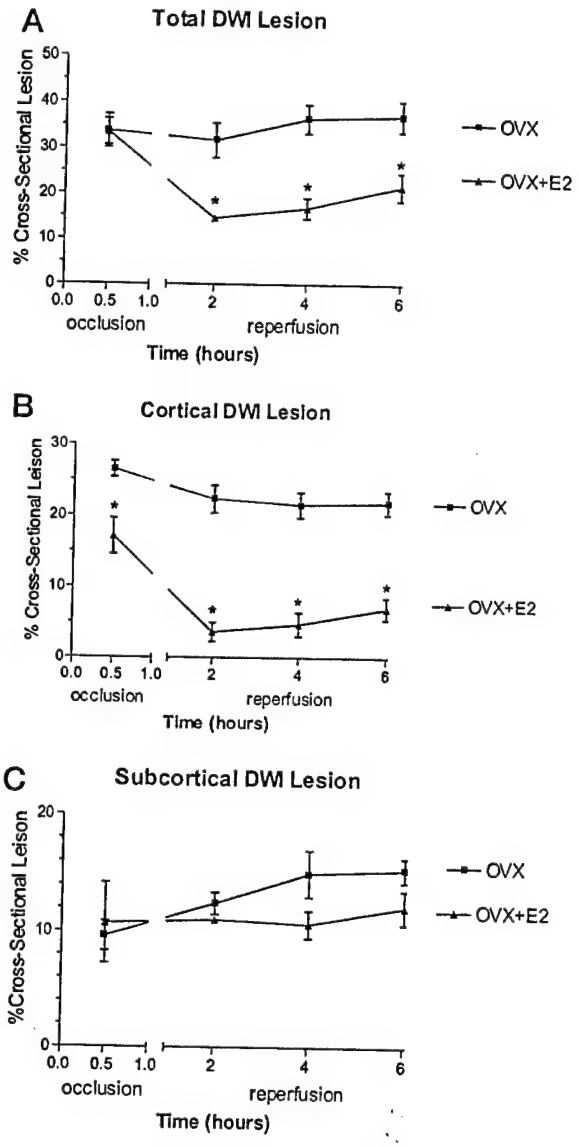

Figure 2. Effects of estrogen on MCAO-induced lesion sizes in female rats assessed by DWI. Serial DWI was applied to assess the total (A), cortical (B), and subcortical (C) lesion sizes in both OVX (n=4) and OVX+E2 (n=3) groups. Average measurements of lesion sizes at 7 and 9 mm were used for statistical analysis because they represented the widest extent of the MCAO lesions. The Mann-Whitney U test was applied to determine the significance of the difference between OVX and OVX+E2 groups. Mean±SEM values are depicted. When SEM is not shown, it is too small to be depicted. *$P<0.05$ vs OVX group.

Results

The mean arterial blood pressure was kept in the normal range during the experiment. The relatively lower Po_2 and relatively higher Pco_2 were due to the anesthesia. There were no significant differences in the determined parameters between OVX and OVX+E2 groups (Table 1).

DWI detected early changes in lesion sizes at 30 minutes into the MCAO. The total MCAO-induced lesion size was similar in both groups (33.7% and 33.5% of the whole hemisphere in the OVX and OVX+E2 groups, respectively) (Figures 1 and 2A) but was larger in cortical regions in the OVX group (26.5% versus 17.1% of OVX+E2) (Figures 1 and 2B). During reper-

TABLE 2. Effects of Estrogen in SIR of Ischemic Lesions Assessed by MRI

	SIR							
	DWI				T2WI			
	Occlusion	2 h	4 h	6 h	Occlusion	2 h	4 h	6 h
Cortex								
OVX	2.35±0.15	2.43±0.25	2.34±0.27	1.81±0.09	0	1.45±0.05	1.67±0.06	1.84±0.05
OVX+E2	1.63±0.12*	1.15±0.38*	1.23±0.40*	1.28±0.26	0	1.26±0.08	1.36±0.08*	1.40±0.11*
Subcortex								
OVX	2.20±0.21	2.38±0.15	2.27±0.22	1.87±0.06	0	1.40±0.04	1.59±0.06	1.76±0.06
OVX+E2	1.51±0.32	1.86±0.05*	2.11±0.11	1.94±0.09	0	1.28±0.01	1.35±0.04	1.52±0.06

Values are mean±SEM. Changes in ischemic lesion intensity were measured by SIR. The Mann-Whitney *U* test was applied to determine the significance of the difference between OVX (n=4) and OVX+E2 (n=3) groups.

*$P<0.05$ vs OVX group.

fusion, the lesion size remained constant in the OVX group but decreased in the OVX+E2 group by 50% to 60% ($P<0.05$) (Figures 1, 2A, and 2B). The size reduction was primarily located in cortical regions (Figures 1, 2A, and 2B).

The intensity of the ischemic lesion, as measured by the signal intensity ratio (SIR), increased at 30 minutes during MCAO in the OVX group (lesion side versus nonlesion side, 2.35 and 2.20 in cortical and subcortical regions, respectively) and reached a plateau during the latter stages of reperfusion until 6 hours of reperfusion, when SIR dropped to 1.81 and 1.87 in cortical and subcortical regions, respectively (Table 2). By comparison, the SIR during MCAO in OVX+E2 animals was reduced (1.63 and 1.51 in cortical and subcortical regions, respectively; $P<0.05$). Reperfusion further attenuated the SIR in cortical but not subcortical regions to 1.28 ($P<0.05$ versus OVX) in the OVX+E2 group (Table 2).

T2WI failed to detect the early vasogenic edema induced by MCAO in either group. During reperfusion, the OVX group demonstrated a continuous increase in lesion sizes in cortical (19.7%, 22.1%, and 24.7% at 2, 4, and 6 hours during reperfusion, respectively) and subcortical (8.3%, 10.1%, and 12.5% at 2, 4, and 6 hours during reperfusion, respectively) regions (Figures 3, 4A, and 4B). In contrast, the OVX+E2 group showed a 70% to 80% decrease in lesion size in cortical regions ($P<0.05$) (Figures 3 and 4B) and a 10% to 25% decrease in subcortical regions (Figures 3 and 4C). T2WI showed less attenuation of SIR by E2 treatment compared with DWI. E2 treatment did not significantly decrease SIR in subcortical regions compared with OVX groups (Table 2) but did cause a 20% decrease ($P<0.05$) in SIR in cortical regions during later reperfusion.

In separate groups of animals, CBF was measured before and during MCAO and 2 times during reperfusion. In both groups, CBF was reduced to approximately 20% of baseline during MCAO. Consistent with our previous observations,[8] CBF gradually recovered during the reperfusion phase, reaching 81% and 97% of baseline in OVX and OVX+E2 groups, respectively, by 24 hours of reperfusion. There were no significant differences between groups at any of the sampling times assessed (Figure 5).

Discussion

Early detection and localization of potentially reversible ischemic damage are crucial for designing and investigating clinical therapeutic interventions against stroke. The present study demonstrates that MRI can provide a wealth of critical information about the initiation, progression, and localization of cerebral ischemic events and herein is used to define the location and the component of the developing ischemic lesion as affected by estrogens. DWI, which is sensitive to the random movement of water molecules, is thought to reveal the early changes associated with stroke-induced cytotoxic edema. On the other hand, conventional T2WI is sensitive to vasogenic edema that occurs later in the pathophysiology of

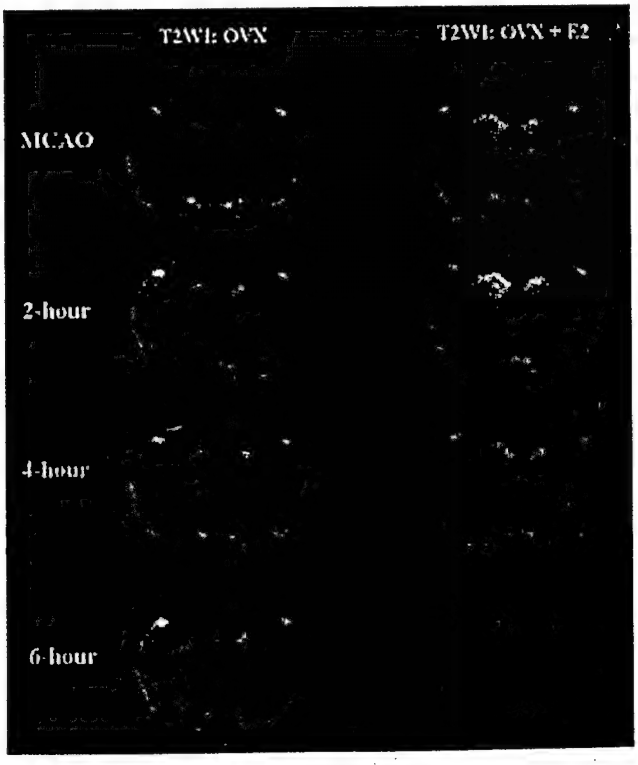

Figure 3. Sequential T2WI from representative OVX and OVX+E2 rats during MCAO and after reperfusion. Two weeks after ovariectomy, female rats were divided into OVX (n=4) and OVX+E2 (n=3) groups. Both groups were then subjected to 1-hour MCAO. Sequential T2WI were then obtained for each animal halfway through the MCAO and at 2, 4, and 6 hours after monofilament removal (reperfusion interval). The imaging sections shown were captured at 9 mm caudal to the end of olfactory bulb.

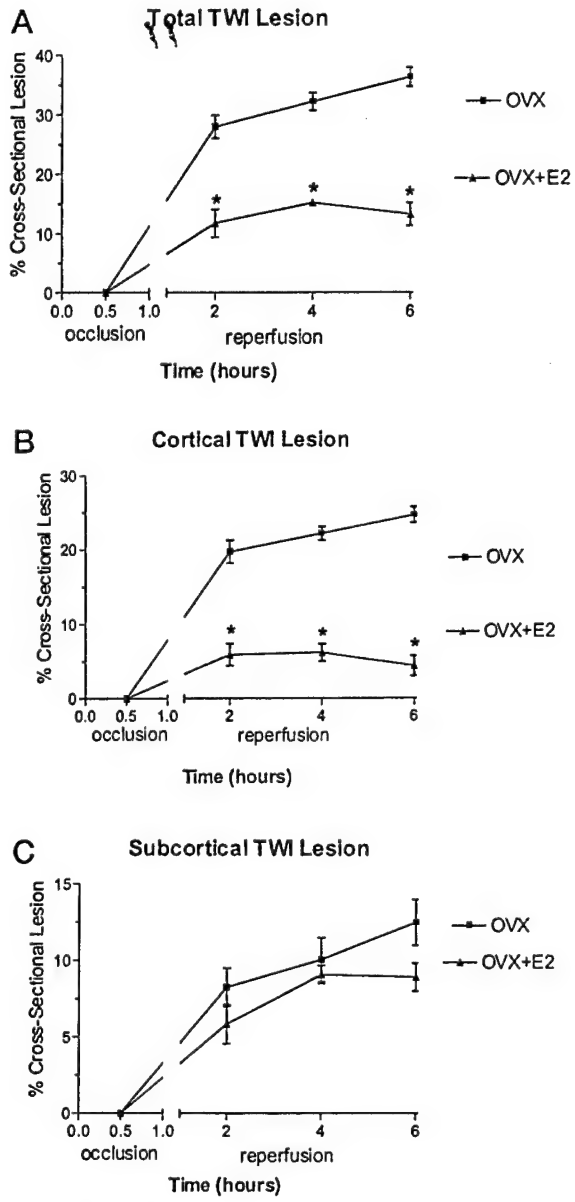

Figure 4. Effects of estrogen on MCAO-induced lesion sizes in female rats assessed by T2WI. Serial T2WI was applied to assess the total (A), cortical (B), and subcortical (C) lesion sizes in both OVX (n=4) and OVX+E2 (n=3) groups. Average measurements of lesion sizes at 7 and 9 mm were used for statistical analysis because they represented the widest extent of the MCAO lesions. The Mann-Whitney *U* test was applied to determine the significance of the difference between OVX and OVX+E2 groups. Mean±SEM values are depicted. When SEM is not shown, it is too small to be depicted. *$P<0.05$ vs OVX group.

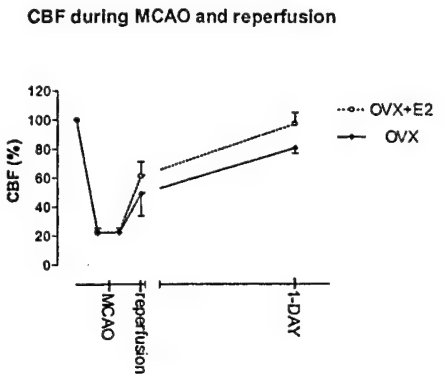

Figure 5. Effects of estrogen on CBF during MCAO and reperfusion in OVX and OVX+E2 rats. Mean±SEM values for OVX (n=5) and OVX+E2 (n=4) rats are shown. There were no differences between the groups at any of the sampling times.

stroke.[16] It can detect subacute ischemic damages, although it fails to show acute ischemic changes.

The early detection of ischemic lesion volumes by DWI is predictive of the clinical severity and outcome of stroke patients.[17,18] In our study DWI provided the earliest detected evidence of cerebral ischemia and was the parameter most affected by E2 treatment. This reduction in DWI changes by E2 treatment can account for most of the observed beneficial effects of estrogen pretreatment.[5–14]

The preferential protection provided by estrogen to cortical versus subcortical tissue could reflect the differential severity of the ischemic damage of these 2 brain regions. The differential severity could result from their different blood supplies. The penumbra of the cortical ischemic region receives collaterals from leptomeningeal anastomosis, as well as from the watersheds between the anterior cerebral artery and the MCA and between the posterior cerebral artery and the MCA, while the cores of the cortical ischemic region and the subcortical region are supplied by terminal arteries of the MCA only.[19] During MCAO, the penumbra may continue to receive limited blood flow from the anterior cerebral artery, while the core and basal ganglion are believed to be more severely occluded. Alternatively, subcortical white matter is more vulnerable to the effects of focal ischemia than cortex.[20] These differences in the vulnerability and blood supply to the core and penumbra area could lead to the difference in neuroprotective effects of estrogen between these 2 areas.

The protective effects of E2 treatment appear to CBF independent. We have previously reported the neuroprotective effects of estradiol in the absence of changes in CBF.[8,15,21] In addition, others have reported a blood flow–independent neuroprotective effect of estrogens in both transient[13] and permanent[10] occlusion models. Consistently in this study, we saw no estrogen-related changes in the extent of decline in CBF during the occlusion and no significant difference in CBF during reperfusion.

E2 treatment appears to exert part of its protective effects by preventing permanent damage associated with reperfusion. Reperfusion causes structural alteration of the Golgi apparatus and compromises the energy supply to brain cells. Hoehn-Berlage et al[22] applied bioluminescence and fluorescence techniques to correlate DWI and energy disturbance during MCAO and found a depletion of ATP in the ischemic core, while the area of tissue acidosis spread beyond the ATP-depleted core region. These findings are consistent with our triphenyltetrazolium chloride staining observation (data not shown) that the white core infarct region is surrounded by the pink ischemic penumbra.[5–14] Additionally, since part of the E2-related improvement in DWI outcome occurs during the reperfusion phase, this delayed effect of the steroid may help to explain the observation that E2 treatment can be delayed until up to 3 hours after the onset of ischemia and lesion size reductions are still observed.[5,15]

Reperfusion of ischemic tissue can produce an influx of oxygen followed by an accumulation of oxygen-derived free radicals.[2] The oxidative stress may damage unsaturated fatty acids in the plasma membrane, which in turn could increase calcium influx into the cell and worsen ischemia-initiated neuronal injuries. We and others have shown that estrogens can attenuate free radical–induced peroxidative damage,[23,24] modulate calcium homeostasis in neurons,[25] and interact with neurotrophins, their receptors, and signaling pathways.[26] All of these effects of estrogen may contribute to its protective effects during reperfusion.

The suggestion that estrogens may have significant protective properties during reperfusion could have profound impact in stroke therapies. Many centers in the United States are now treating stroke acutely, using thrombolytic agents to dissolve the offending clot. Clinical trials have demonstrated a significant clinical improvement in such patients, especially when the treatment is delivered within 3 hours of stroke onset. Reopening an occluded intracranial vessel, however, is not without serious risks, which may include acute or delayed intracerebral hemorrhage, reperfusion hyperemia, and progression to infarction despite a patent lumen. The identification of an agent that can protect against such mechanisms, if delivered before or early after the vessel is reopened, could minimize an otherwise preprogrammed infarction and perhaps also positively influence hemorrhagic risks by stabilizing energy metabolism in vascular endothelium.

The present study suggests that estrogens are good candidates for producing such effects. During reperfusion, E2 treatment dramatically decreases ischemic lesion sizes and intensities, as demonstrated by both DWI and T2WI, and these decreases are almost exclusively located in cortical regions. In our previous study we found that a single dose (100 μg/kg) of E2 could increase the serum E2 concentration to physiological level in rats, producing levels sufficient for neuroprotection.[6] These findings are also consistent with a study showing that OVX can increase the ischemic lesion volume in a focal stroke model.[5]

In summary, we applied MRI techniques to demonstrate the temporal and spatial ischemic changes in a focal ischemic animal model. We have demonstrated that estrogens selectively protect cortical tissue from ischemic damage and that this protection is exerted during both the occlusion and reperfusion phases of ischemia. This study suggests that estrogen could have direct clinical applications by protecting against thrombolytic-induced reperfusion injury.

Acknowledgments

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Appendix B

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The Nonfeminizing Enantiomer of 17β-Estradiol Exerts Protective Effects in Neuronal Cultures and a Rat Model of Cerebral Ischemia*

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ABSTRACT

Estrogens are potent neuroprotective compounds in a variety of animal and cell culture models, and data indicate that estrogen receptor (ER)-mediated gene transcription is not required for some of these effects. To further address the requirement for an ER in estrogen enhancement of neuronal survival, we assessed the enantiomer of 17β-estradiol (*Ent*-E_2), which has identical chemical properties but interacts only weakly with known ERs, for neuroprotective efficacy. *Ent*-E_2 was both as potent and efficacious as 17β-estradiol in attenuating oxidative stress-induced death in HT-22 cells, a murine hippocampal cell line. Further, *Ent*-E_2 completely attenuated H_2O_2 toxicity in human SK-N-SH neuroblastoma cells at a 10 nM concentration. In a rodent model of focal ischemia, 17β-estradiol (100 μg/kg) or *Ent*-E_2 (100 μg/kg), injected 2 h before middle cerebral artery occlusion, resulted in a 60 and 61% reduction in lesion volume, respectively. *Ent*-E_2, at the doses effective in this study, did not stimulate uterine growth or vaginal opening in juvenile female rats when administered daily for 3 days. These data indicate that the neuroprotective effects of estrogens, both *in vitro* and *in vivo*, can be disassociated from the peripheral estrogenic actions. (*Endocrinology* **142:** 400–406, 2001)

EPIDEMIOLOGICAL STUDIES ASSOCIATE postmenopausal estrogen replacement therapy with several beneficial neurological outcomes, including a reduction in incidence of Alzheimer's disease (1, 2), Parkinson's disease (3, 4), and death from stroke (5, 6). These effects may be mediated, in part, by estrogen-mediated enhancement of neuronal survival. The neuroprotective effects of estrogens, specifically the potent 17β-estradiol (βE_2), have been widely described in neuronal cultures against toxicities, including growth factor deprivation, glutamate toxicity, and oxidative stress (for review, see Ref. 7). Similarly, in rodents, βE_2 has been shown to attenuate neuronal loss after cerebral ischemia (8–10), kainic acid administration (11), and physical injury (12).

The role of estrogen receptor (ER)-dependent transcription in estrogen's neuroprotective activity is controversial (for review, see Ref. 7). The neuroprotective activity of βE_2 in culture models is attenuated by the ER antagonists tamoxifen or ICI 182,780 in some studies (13–15); however, others report no effect of these same ER antagonists on βE_2-mediated neuroprotection (16–20). Our laboratory (16, 21, 22) and others (17, 23) have reported equipotent (to βE_2) neuroprotective efficacy of 17α-estradiol, which only weakly activates ER-dependent gene transcription (24), implicating mechanisms other than ER-mediated transcription in estrogen-mediated protection of neuronal cultures. In mouse models of cerebral ischemia, the data are equally inconclusive. Sampei *et al.* (25) report no difference in total lesion size between wild-type and ERα-deficient mice. However, ICI 182,780 administration increases striatal lesion volume in the wild-type mouse (26). ICI 182,780 administration did not alter neocortical lesion volume in this study. It is important to note that, although protection of neocortical areas are consistently reported with βE_2 treatment (8–10), βE_2-mediated protection of striatal infarct is not consistently reported (9).

The present study addresses the requirement for ER-dependent transcription in the neuroprotective effects of estrogens, both *in vitro* and *in vivo*, using a novel enantiomer strategy. Ent-17β-estradiol (*Ent*-E_2), the enantiomer of the naturally occurring βE_2 (Fig. 1), has identical physiochemical properties as βE_2 except for interactions with other stereospecific molecules such as ERs. *Ent*-E is reported to interact only weakly with uterine-derived ERs (27, 28) and lacks estrogenic effects on reproductive tissues in rodents (29–31). Some reports indicate that *Ent*-E_2 exerts slight antiuterotrophic activity and can antagonize the uterotrophic effects of βE_2 (32, 33). In contrast, *Ent*-E_2 has been reported to elicit alterations in lipid profiles identical to βE_2, with similar potency (34). Further, *Ent*-E_2 is not enzymatically converted to βE_2 (35) and, therefore, is more adequately suited than 17α-estradiol for evaluating the role of ERs in estrogen-mediated neuroprotection. This study evaluates the neuroprotective effects of *Ent*-E_2, both in culture models of oxidative stress and in a rat transient focal ischemia model and, further,

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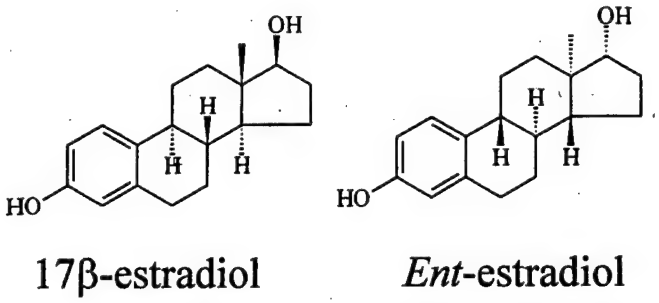


FIG. 1. Structure of the naturally occurring βE_2 and the nonnaturally occurring *Ent*-E_2.

determines whether *Ent*-E_2 can exert neuroprotective effects in the absence of stimulation of peripheral estrogen-responsive tissues.

Materials and Methods

Steroids

βE_2 was purchased from Steraloids, Inc. (Wilton, NH). *Ent*-E_2 was synthesized from the known starting material, [3*R*-(3α,3aα,9aα,9bβ)]-3-(1,1-dimethylethoxy)-1,2,3,3a,4,5,8,9,9a,9b-decahydro-3a-methyl-6-[2-(2-methyl-1,3-dioxalan-2-yl)ethyl-7*H*-benz[*e*]inden-7-one (Chemical Abstracts Registry Number 139973-49-2), which was prepared by a multistep synthetic pathway as described in the literature (36). This compound was then converted in either of two ways (method A or method B) to *Ent*-19-nortestosterone (Chemical Abstracts Registry Number 4091-86-5). In the first step of method A, the double bond is reduced, using lithium in liquid ammonia, and the resulting tricyclic compound is cyclized to *Ent*-19-nortestosterone in the second step. In the first step of method B, the double bond is reduced by catalytic hydrogenation, and the resulting tricyclic compound is again cyclized to *Ent*-19-nortestosterone in the second step. Method B has been previously used to prepare 19-nortestosterone (37). The hydroxy group of *Ent*-19-nortestosterone is then esterified, and the A-ring of the steroid is aromatized using $CuBr_2$ in acetonitrile. This reaction has been reported previously for the conversion of 19-nortestosterone, 17-acetate to 17β-estradiol, 17-acetate (38). Finally, the 17-acetate group is removed by saponification to give *Ent*-E_2 (Chemical Abstracts Registry Number 3736-22-9). The structure of *Ent*-E_2 was proven by experimental data, which showed that the compound had the same melting point (176-177 C), infrared absorption spectra (3449, 3246, 2936, 2864, 1611, 1587, 1500, 1450, 1283, 1250, 1057, 1012, 930, 874 cm^{-1}), ^1H NMR [(300 MHz, CD_3OD) δ 7.06 (1 H, d, J = 8.7 Hz), 6.54-6.46 (2 H, m), 3.64 (1 H, t, J = 8.4 Hz), 0.75 (3 H)] and ^{13}C NMR [(75 MHZ, CD_3OD) δ 156.07, 138.98, 132.80, 127.32, 116.18, 113.85, 82.57, 51.32, 45.34, 44.36, 40.50, 38.01, 30.67 (2 × C). 28.48, 27.56, 23.99, 11.62) spectra but opposite optical rotation ($[\alpha]_D^{28}$ = −71.2 (c = 0.99, CH3OH)) as βE_2.

Steroids were initially dissolved in ethanol at a 10 mM concentration and then diluted to the appropriate concentration in culture media or assay buffer for cell culture or *ex vivo* assays, respectively. Steroids were dissolved in corn oil at the concentration necessary to yield the indicated dose in 1 ml/kg injection volume for rodent studies.

Cell culture

SK-N-SH human neuroblastoma cells were obtained from ATCC-(Manassas, VA), and HT-22 cells (immortalized hippocampal neurons of murine origin) were a generous gift of Dr. David Schubert (The Salk Institute, San Diego, CA). Cells were maintained in RMPI-1640 and DMEM media (Life Technologies, Inc., Gaithersburg, MD), respectively, supplemented with 10% charcoal/dextran-stripped FBS (HyClone Laboratories, Inc., Logan, UT) and 200 μg/ml gentamycin, according to standard culture conditions.

Cells were plated, 24 h before initiation of experiment, at a density of 20,000 cells/well (SK-N-SH cells) or 5,000 cells/well (HT-22 cells), in both clear- and white-bottomed Nunc 96-well plates (Fisher Scientific, Orlando, FL). Steroids were added at concentrations ranging from 0.1 nM to 10 μM, either 2 or 24 h before exposure to either glutamate (5 mM) or H_2O_2 (3-60 μM). Ethanol was used at concentrations of 0.001-0.1% vol/vol as a vehicle control. These concentrations of ethanol had no discernible effect on cell viability. After 24 h of toxin exposure, cells were rinsed with PBS, pH 7.4, and viability was assessed by the addition of 1 μM calcein AM (Molecular Probes, Inc., Eugene, OR) and 1 μg/ml propidium iodide (Sigma, St. Louis, MO) in PBS for 15 min. Calcein AM fluorescence was determined at an excitation of 485 nm and an emission of 538 nm. Percent viability was calculated by normalization of all values to the toxin-free control group (= 100%). Percent protection was calculated as the difference between each experimental value and the average of the toxin-only group normalized to the difference between the toxin-free control and toxin-only groups (= 100% protection). Cells that had been lysed by addition of 1% SDS were used for blank readings. Staining was visualized using a fluorescent Nikon (Melville, NY) microscope, and cells were photographed for qualitative documentation.

Animals

Female Sprague Dawley rats (Harlan, Indianapolis, IN) were housed in pairs in hanging, stainless steel cages in a temperature-controlled room (25 ± 1 C) with a daily light cycle (on from 0700 to 1900 h daily). All rats had free access to laboratory chow and tap water. All procedures performed on animals were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Florida before the initiation of the study.

Ovariectomy

Female Sprague Dawley rats (220-225 g BW) were given 3-5 days to acclimate, then were bilaterally ovariectomized using a dorsal approach. Animals were anesthetized with methoxyflurane (Pitman Moore, Inc., Crossing, NJ) inhalant anesthesia. A small (1 cm) cut was made through the skin, facia, and muscle. The ovaries were externalized, clipped, and removed; then the muscle, facia, and skin were sutured closed. Ovariectomy was performed 2 weeks before experiments.

Middle cerebral artery (MCA) occlusion

Either oil vehicle or 100 μg/kg βE_2 or *Ent*-E_2 was administered by sc injection, 2 h before the onset of MCA occlusion. Animals were anesthetized by ip injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). MCA occlusion was performed as previously described (8). Briefly, the left common carotid artery, external carotid artery, and internal carotid artery were exposed through a midline cervical incision. A 3-0 monofilament suture was introduced into the internal carotid artery lumen and gently advanced until resistance was felt. The suture was kept in place for 60 min and then withdrawn to allow MCA reperfusion. The procedure was performed within 20 min, with minimal bleeding. Rectal temperature was maintained between 36.5 and 37.0 C during the entire procedure.

Animals were decapitated and the brain removed 24 h after onset of MCA occlusion. The brain was then dissected coronally into 2-mm sections using a metallic brain matrix (ASI Instruments, Inc., Warren, MI). The sections located 3, 5, 7, 9, and 11 mm posterior to the tip of the olfactory bulb were stained by incubation in a 2% solution of 2,3,5-triphenyltetrazolium chloride in a 0.9% saline solution at 37 C for 30 min. Slices were then fixed in 10% formalin and photographed, and the ischemic lesion area was determined for each slice using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). Percent ischemic lesion area was calculated as the sum of the ischemic lesion area for the five slices divided by the total cross-sectional area of these five slices.

Plasma levels of βE_2

Ovariectomized female Sprague Dawley rats were injected sc with either oil vehicle or 100 μg/ml βE_2 or *Ent*-E_2. Blood samples were obtained by cardiac puncture, 5 min before injection or 1 h, 4 h, or 24 h post injection. Plasma was stored at −20 C, until assayed using the ultrasensitive βE_2 RIA kit from Diagnostic Systems Laboratories, Inc. (Los Angeles, CA) according to the manufacturer's instructions. *Ent*-E_2 showed no cross-reactivity with the RIA at concentrations up to 10 μM.

Uterotrophic assay

Juvenile (25 days old) female Sprague Dawley rats were injected sc with oil, βE_2 (0.01–1 μg/rat), or *Ent*-E_2 (1–100 μg/rat) daily (at 0830 h) for 3 days. On the fourth day, the rats were killed using methoxyflurane, and the uteri were excised. Extraneous tissue was gently removed from the uteri before wet weight was determined. Vaginal opening was assessed before uterine removal.

Ligand competition of ER binding

5 nM [2,4,6,7-^{3}H]-βE_2 (specific activity, 84.1 Ci/mmol; Amersham Pharmacia Biotech, Piscataway, NJ) and 400 pM recombinant human ERα or ERβ (Affinity BioReagents, Inc., Golden, CO) were incubated in ER binding buffer (20 mM Tris, 1 mM EDTA, 400 mM KCl, 1 mM dithiothreitol, 10% glycerol, 0.1% BSA, pH 7.8) for 1 h at 25 C either with no added steroid (total binding), 1.2 μM diethylstilbestrol (nonspecific binding), or 0.1 nM–10 μM βE_2 or *Ent*-E_2. Bound and unbound radioligand were separated using Sephadex G-25 (Amersham Pharmacia Biotech) columns (1.5 ml bed volume) with a 1-ml elution volume. Ten milliliters of scintillation fluid was added, and counts were determined. This method resulted in greater than 90% receptor recovery and less than 15% nonspecific binding.

Brain membrane oxidation

The brain was removed from ovariectomized female Sprague Dawley rat, and the neocortex was homogenized in ice-cold Tris buffer (100 mM, pH 7.4) with 1% Triton X-100 using a Teflon/glass tissue homogenizer. The homogenate was centrifuged at 2,000 rpm for 10 min. The resulting supernatant was incubated with βE_2 or *Ent*-E_2 at concentrations ranging from 0.1–100 μM for 30 min at 37 C. $FeSO_4$ was then added to a final concentration of 200 μM and incubated for an additional 30 min at 37 C. Butylated hydroxytoluene (100 μM) and diethylenetriaminepentaacetic acid (100 μM) were then added. 2-thiobarbituric acid reactive products (TBARs) were immediately determined by addition of 0.5% 2-thiobarbituric acid, 3.125% trichloroacetic acid, and 0.2 N HCl; and incubation was performed, at 95 C for 1 h. Samples were centrifuged at 10,000 rpm for 10 min, and the absorbance of the supernatant at 532 nm was determined.

Statistical analysis

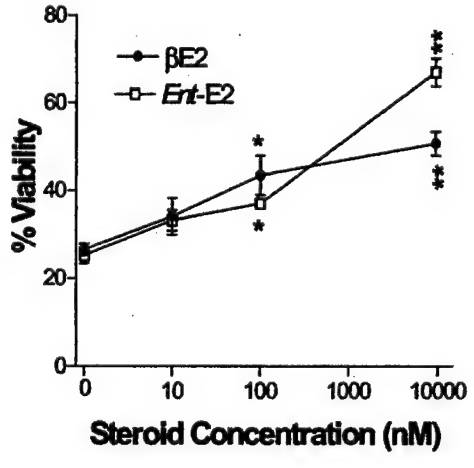
All data are presented as mean ± SEM. Comparison of ischemic lesion volume was performed using a one-way ANOVA with a Kruskal-Wallis test for planned comparisons between groups. For all other experiments, the significance of differences among groups was determined by one-way ANOVA with a Tukey's multiple-comparisons test for planned comparisons between groups when a significant difference was detected. For all tests, $P < 0.05$ was considered significant.

Results

Ent-E_2 attenuates oxidative stress-induced death in neuronal cultures

HT-22 cells, transformed hippocampal neurons, are sensitive to glutamate toxicity via a mechanism that involves glutathione depletion and the resulting oxidative stress (39). Exposure of HT-22 cells to 10 mM glutamate caused a 70–75% reduction in neuronal viability, by 24 h of exposure (Fig. 2). As previously reported (40), βE_2 treatment, commencing 2 h before glutamate exposure, conferred significant protection in this model, with a 10,000 nM concentration protecting 35 ± 4% of the cells. *Ent*-E_2, performed similarly in this model of neuroprotection, with 100 nM and 10,000 nM *Ent*-E_2 protecting 16 ± 2% and 56 ± 4% of HT-22 cells, respectively.

In another model of oxidative stress, both βE_2 and *Ent*-E_2 significantly attenuated H_2O_2-induced toxicity in HT-22 cells (Fig. 3). H_2O_2 exposure resulted in a concentration-dependent toxicity in HT-22 cells with a 30-μM concentration resulting in 21 ± 5% reduction in viability (Fig. 3) and a 60-μM concentration resulting in a 97 ± 8% reduction (data not shown);10 nM of either βE_2 or *Ent*-E_2 completely attenuated the toxicity of 30 μM H_2O_2 and protected 48 ± 14% or 63 ± 8% of the cells from 40 μM H_2O_2 toxicity, respectively (Fig. 3). No protection was seen with the 10 nM concentration of either steroid at H_2O_2 concentrations greater than 40 μM



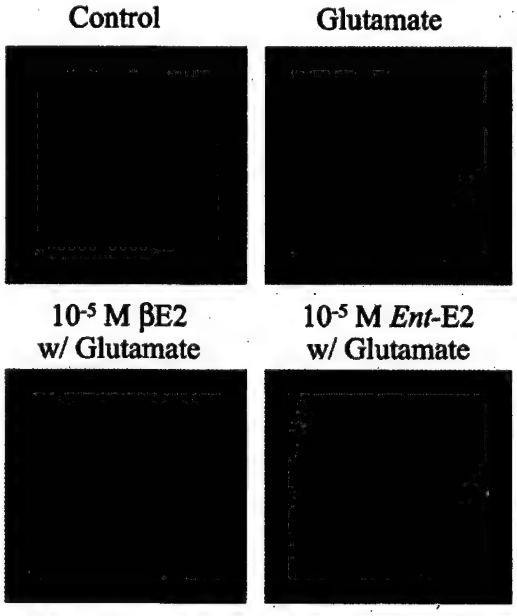


FIG. 2. Effects of βE_2 and *Ent*-E_2 on glutamate toxicity in HT-22 cells. The indicated concentration of steroid was added 2 h before the addition of glutamate (5 mM), and viability was assessed 24 h later using calcein AM fluorescence. Relative fluorescence units were normalized to the respective toxin-free group as 100% viability. Shown is mean ± SEM for four to eight wells, representative of at least two individual experiments. *, $P < 0.05$; **, $P < 0.01$ *vs.* toxin only group. Pictured are representative fields stained with calcein AM (*green*) and propidium iodide (*red*).

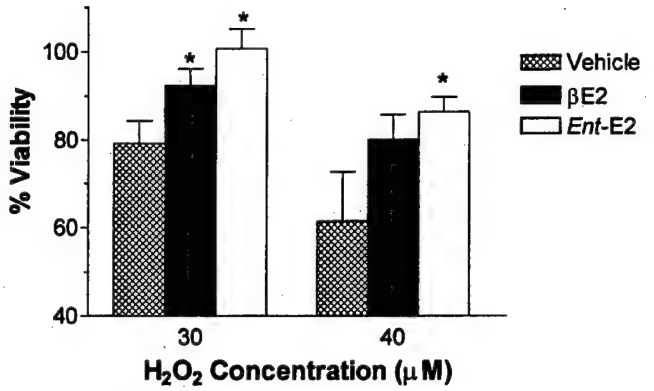


FIG. 3. Effects of βE_2 and *Ent*-E_2 on H_2O_2 toxicity in HT-22 cells. The steroid (10 nM) was added to HT-22 cells 2 h before the addition of the indicated concentration of H_2O_2. Viability was assessed 24 h later using calcein AM fluorescence. Relative fluorescence units were normalized to the respective toxin-free group as 0% reduction in viability. Shown is mean ± SEM for four wells, representative of at least two individual experiments. *, $P < 0.05$ *vs.* toxin-only group.

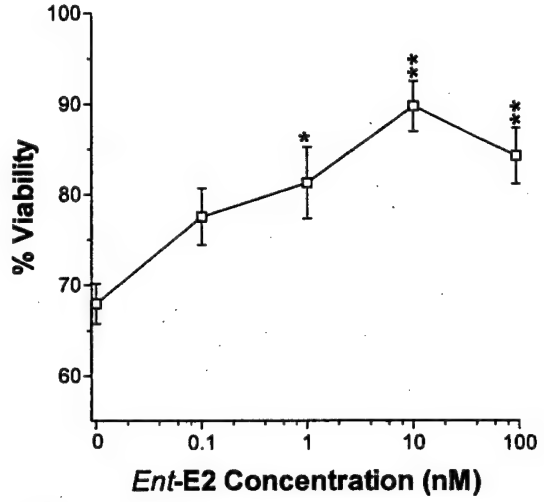


FIG. 4. Effect of *Ent*-E_2 on H_2O_2 toxicity in SK-N-SH cells. The indicated concentration of *Ent*-E_2 was added 24 h before the addition of 3 μM H_2O_2. Viability was assessed 24 h later using calcein AM fluorescence. Relative fluorescence units were normalized to the respective toxin-free group as 100% viability. Shown is mean ± SEM for 3–4 wells, representative of at least two individual experiments. *, $P < 0.05$; **, $P < 0.001$ *vs.* the toxin-only group.

(data not shown). SK-N-SH cells were more sensitive than HT-22 cells to the toxic effects of H_2O_2 exposure, with 3 μM H_2O_2 reducing SK-N-SH cell viability by 32 ± 2% (Fig. 4). This kill was significantly attenuated by *Ent*-E_2, with a 1 nM concentration conferring 30 ± 9% protection (Fig. 4). In other studies, 1 nM βE_2 prevented 40 ± 5% of H_2O_2-induced toxicity in SK-N-SH cells (data not shown). Neither steroid exerted protective effects in these low nM concentration ranges with higher concentrations of H_2O_2 (data not shown).

Ent-E_2 reduces ischemic lesion size after transient MCA occlusion

Transient (1 h) occlusion of the MCA resulted in an average lesion area of 13 ± 2%, with the lesion localized primarily in the parietal cortex and basal ganglia (Fig. 5). Comparable with our previous reports (8), sc injection of βE_2, 2 h before onset of ischemia, reduced total lesion area by 60 ± 13%. Administration of *Ent*-E_2 similarly reduced total ischemic area by 60 ± 12%. This estrogen-mediated protection was observed in both neocortical and subcortical/allocortical regions of the ischemic infarct. βE_2 and *Ent*-E_2 reduced neocortical lesion size by 77 ± 11% and 59 ± 12%, respectively. Similarly, subcortical/allocortical lesion volume was reduced by 48 ± 8% and 47 ± 7% by βE_2 and *Ent*-E_2 administration, respectively. The subcortical/allocortical ischemic area includes regions of the caudate/putamen, hypothalamus, and hippocampus.

The protective effects of *Ent*-E_2 in this model are not attributable to conversion of *Ent*-E_2 to the more estrogenically potent βE_2. Plasma βE_2 levels after *Ent*-E_2 administration did not change from the preinjection baseline of 0.05 ± 0.01 nM (Fig. 6). In contrast, sc injection of βE_2 resulted in a rapid rise in plasma βE_2 levels, with values of 5.16 ± 0.94 nM within 1 h, and returned to near baseline (0.24 ± 0.08 nM) by 24 h.

Ent-E_2 is a weak ER agonist/antagonist

Daily administration of βE_2 for 3 days caused a dose-dependent increase in uterine wet weight, with a 1-μg/rat dose (average dose of 13.8 μg/kg) increasing wet uterine weight by 2-fold (Fig. 7). By contrast, *Ent*-E_2, at doses of 1–10 μg/rat, had no effect on uterine wet weight. At a dose of 100 μg/rat (average dose of 1400 μg/kg), *Ent*-E_2 exerted a slight antiuterotrophic effect, decreasing uterine wet weight by 23 ± 3%. *Ent*-E_2 also slightly antagonized the uterotrophic effects of 1 μg/rat βE_2, with a 100 μg/rat dose reducing the uterotrophic effect of βE_2 by 27 ± 8%. These results are comparable with previous reports in immature mice, where *Ent*-E_2 (doses of about 1200 μg/kg) exerted antiuterotrophic effects (29) and *Ent*-E_2 antagonized the uterotrophic effects of βE_2 when *Ent*-E_2 was present in a 100-fold excess (32).

Daily injections of βE_2 (1 μg/rat) induced vaginal opening in 100% of the animals examined (Table 1). *Ent*-E_2 exerted mixed agonist/antagonist effects on vaginal opening, with a 100-μg/rat dose causing vaginal opening in 50% of the juvenile rats. This dose of *Ent*-E_2 prevented βE_2-induced vaginal opening in 40% of the rats. No change in body weight was observed with administration of βE_2, *Ent*-E_2, or combinations thereof. BW of the juvenile rats averaged 72 ± 1 g.

In competition binding experiments, *Ent*-E_2 showed weak binding to both known ERs, with 4.2% and 6.3% of the relative binding affinity of βE_2 to ERα and ERβ, respectively. *Ent*-E_2 has been previously reported to have 0.9–6% of βE_2's relative binding affinity to cytosolic uterine ERs (27, 28).

Ent-E_2 can attenuate brain lipid oxidation ex vivo

Because estrogens have been previously reported to reduce oxidative damage to brain lipids (41–43), we examined the potency of both βE_2 and *Ent*-E_2 in an *ex vivo* assay of brain membrane oxidation. Thirty-minute incubation of the neocortical homogenate resulted in a 16-fold increase in TBAR formation. βE_2 and *Ent*-E_2 were equipotent in the attenuation of $FeSO_4$-induced lipid oxidation, as determined by TBAR formation (Fig. 8), with a 50-μM concentration of either steroid significantly attenuating $FeSO_4$-induced TBAR formation.

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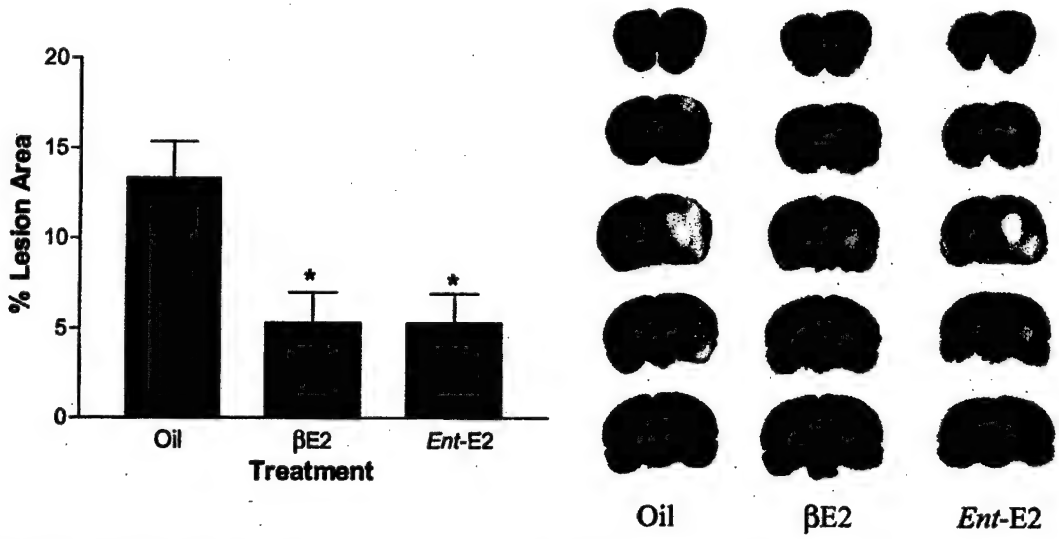

FIG. 5. Effects of βE_2 and *Ent*-E_2 on MCA occlusion-induced lesion volume in ovariectomized female rats. Rats were ovariectomized 2 weeks before occlusion, and steroids were administered, by sc injection, 2 h before onset of focal ischemia. After 1 h of MCA occlusion and 23 h of reperfusion, the brains were removed, and 2-mm slices were prepared at 3, 5, 7, 9, and 11 mm posterior to the olfactory bulb. Lesion area was determined by 2,3,5-triphenyltetrazolium chloride staining. Graphed is mean ± SEM for six rats per group. *, $P < 0.05$ *vs.* vehicle-treated rats. Pictured are representative slices for each treatment group.

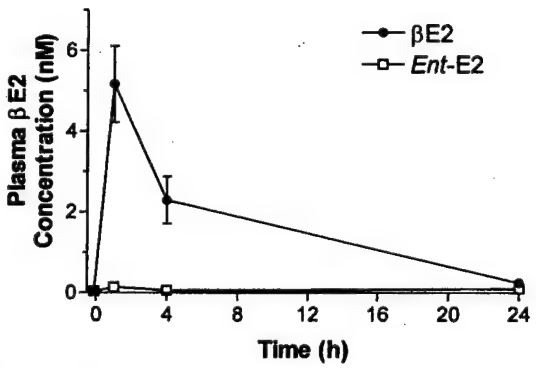

FIG. 6. Plasma βE_2 levels after βE_2 and *Ent*-E_2 administration. Ovariectomized female Sprague Dawley rats were injected sc with 100 μg/kg of either βE_2 or *Ent*-E_2. Blood was drawn by cardiac puncture, either 5 min before injection, 2 h post injection, 4 h post injection, or 24 h post injection. Plasma was collected, and βE_2 concentration was determined by RIA. Plasma βE_2 concentration is given in nM units: 1 nM = 272 pg/ml. Shown are mean ± SEM for three rats per group.

Discussion

Ent-E_2 was both as potent and efficacious as βE_2 in culture models of neuroprotection; and further, *Ent*-E_2 reduced ischemic lesion area after MCA occlusion to the same degree as βE_2. In contrast, *Ent*-E_2 showed only minimal binding affinity for either known ER, was more than 100-fold less potent than βE_2 in exerting effects on uterine growth or vaginal opening, and had weak antiuterotrophic effects. These data indicate that the neuroprotective effects of estrogens can occur without stimulation of peripheral estrogen-responsive tissues.

The neuroprotective effects of *Ent*-E_2 are not likely caused by conversion to the more estrogenically potent βE_2, because the conversion requires isomerization of five individual chiral carbons. Isomerization of the 17-hydroxy group could be facilitated by 17β-hydroxysteroid dehydrogenase; however, *Ent*-E_2 is not a substrate for this enzyme (35). Further, there was no detectable increase in plasma βE_2 levels during 24 h after sc injection of *Ent*-E_2 in female rats, indicating that *Ent*-E_2 is itself neuroprotective.

The minimal neuroprotective concentration of both βE_2 and *Ent*-E_2 varied with the cell culture model used. High physiological concentrations (low nM) were sufficient to attenuate H_2O_2-induced toxicity in SK-N-SH cells, but significantly higher supraphysiological concentrations (low μM) were required to lessen glutamate toxicity in HT-22 cells. This difference in the neuroprotective potency of estrogens between these models may be attributable to a number of factors, including differences in culture media and differences in toxicity. Further, the concentration of steroid required for protection may depend on the degree of insult, because low concentrations of *Ent*-E_2 or βE_2 did not protect SK-N-SH or HT-22 cells from H_2O_2 exposure if viability was reduced by more than 70% (P. S. Green and J. W. Simpkins, unpublished observations).

The high doses of *Ent*-E_2 used in some, but not all, of the experiments in the present report could show appreciable ER binding; however, this does not adequately explain the equipotent neuroprotection conferred by the enantiomer. The 16- to 100-fold-lower affinity of *Ent*-E_2 for the known ERs (this report, and Refs. 27 and 28) would be apparent as a similar 16- to 100-fold-lower potency in effects mediated by either ERα or ERβ. This potency difference was seen in uterotrophic and vaginal opening responses but not in assays of neuroprotection. Regardless of the minimum dose required for neuroprotection in each model, *Ent*-E_2 attenuated neuronal death with a potency equivalent to that of βE_2. This result indicates that enantiospecific interactions between estrogens and other cellular molecules are not required for the neuroprotective actions of estrogens.

Several lines of evidence connote that the neuroprotective effects of estrogens do not require ER-dependent gene tran-

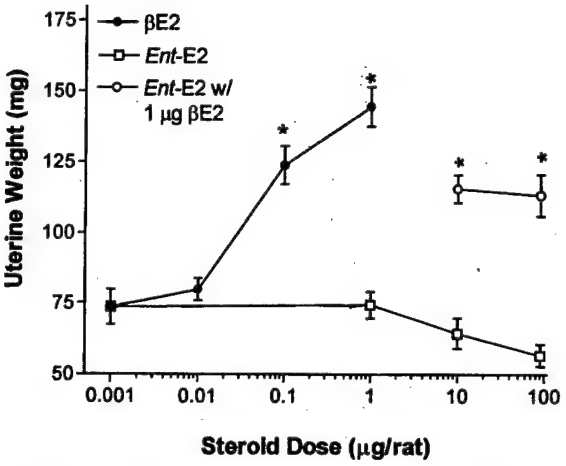

FIG. 7. Effects of βE_2 and *Ent*-E_2 on uterine wet weight in juvenile rats. Twenty-five-day-old female rats were injected sc with the indicated dose of βE_2 or *Ent*-E_2, or concurrent administration of the indicated dose of *Ent*-E_2 with 1 μg/rat βE_2 daily for 3 days. On day 4, the uteri were resected and weighed. Shown are mean ± SEM for three to nine rats per group. *, $P < 0.05$ *vs.* oil injection.

TABLE 1. Effects of βE_2 and Ent-E_2 on vaginal opening in juvenile female rats

Ent-E_2 Dose (μg/rat)	Number with vaginal opening	
	Without βE_2	With 1 μg/rat βE_2
0	0 of 4	5 of 5
10	1 of 4	3 of 4
100	2 of 4	3 of 5

Twenty-five-day-old female Sprague-Dawley rats were injected sc with the indicated dose of Ent-E_2 with or without concurrent administration of 1 μg/kg βE_2, daily for 3 days. On day 4, vaginal opening was assessed.

scription, including potent neuroprotective efficacy of several nonfeminizing estrogens including *Ent*-E_2 (Figs. 2–5) and 17α-estradiol (16, 21–23). Further, functional ERs have not been found in either HT-22 cells (40, 44) or SK-N-SH cells (22), although this study (Figs. 2–4) and others (16, 21–23, 40, 44) demonstrate estrogen-mediated protection of these neuronal cell lines. Similarly, βE_2-mediated protection can occur in the presence of ER antagonists (16–20). Together, these findings, though not excluding a role for ERs in neuroprotection, implicate cellular mechanisms other than classical ER activity in the neuroprotective effects of estrogens.

Antioxidant effects have been proposed as one mechanism for the neuroprotective effects of estrogens (19). Interestingly, the structure-activity relationship for the antioxidant effects of estrogens (42) is identical to the structure-activity relationship for the neuroprotective effects (22, 23). Further, it has been reported that the concentrations of βE_2 that are capable of exerting *ex vivo* antioxidant effects were required for neuroprotective effects (19). βE_2 attenuation of lipid peroxidation has been shown to require μM concentrations (19, 41–43). The neuroprotective concentration for βE_2 in culture models ranges from 0.1 nM (21, 45) to 50 μM (18). In this study, *ex vivo* antioxidant effects of βE_2 and *Ent*-E_2 required a minimum concentration of 50 μM, whereas neuroprotective effects were seen at much lower concentrations.

Neuronal effects of estrogens with weak ER agonist ac-

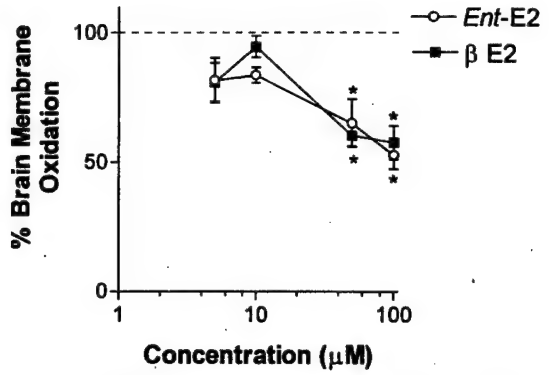

FIG. 8. βE_2 and *Ent*-E_2 inhibit $FeSO_4$-induced lipid oxidation in a rat brain homogenate. Homogenate was prepared from the neocortical tissue of an ovariectomized female Sprague Dawley rat. Homogenate was incubated with the indicated concentration of steroid for 30 min, and then oxidized by a 30-min incubation with 200 μM $FeSO_4$ at 37 C. The extent of lipid oxidation was determined by TBAR formation. Data were normalized to $FeSO_4$-only group as 100% oxidation. Shown are mean ± SEM for three samples per group. *, $P < 0.05$ *vs.* $FeSO_4$-only group.

tivity are being increasingly described. The classically inactive estrogen, 17α-estradiol, has been shown to be neuroprotective in both culture (16, 21–23) and MCA occlusion models (8). Similarly, the weak ER agonist dihydroequilin has been shown to exert neurotrophic effects in cultured neurons (46). The cellular mechanisms for these effects of weak ER agonists is not known; however, several cellular effects of 17α-estradiol have been described. Exposure to αE_2 can activate the MAP kinase pathway (47), and this pathway is implicated in βE_2-mediated neuroprotection (14). In addition, αE_2 has also been shown to have several other direct effects on neurons, including modulation of the mitochondrial $Na+/K^+$-ATPase activity (48), alteration of membrane fluidity (49), and inhibition of toxin-induced activation of NFκB (P. S. Green and J. W. Simpkins, unpublished observations). It is unknown whether *Ent*-E_2 can also interact with any of these cellular pathways.

A profusion of data indicates that estrogens enhance the survival of neurons both *in vitro* and *in vivo*, suggesting that estrogens may be useful in the treatment of neurodegenerative disease or acute neuronal death. Estrogens, such as *Ent*-E_2, may offer the beneficial neuroprotective effects of estrogens without the complicating peripheral estrogenic actions and could be useful in both men and women for whom estrogen therapy is contraindicated.

Acknowledgments

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Appendix C

Neuroprotective Effects of a Novel Non–Receptor-Binding Estrogen Analogue

In Vitro and In Vivo Analysis

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Background and Purpose—Although estrogens are neuroprotective, hormonal effects limit their clinical application. Estrogen analogues with neuroprotective function but lacking hormonal properties would be more attractive. The present study was undertaken to determine the neuroprotective effects of a novel 2-adamantyl estrogen analogue, ZYC3.

Methods—Cytotoxicity was induced in HT-22 cells by 10 mmol/L glutamate. 17β-Estradiol (E2) or ZYC3 was added immediately before the exposure to glutamate. Cell viability was determined by calcein assay. The binding of E2 and ZYC3 to human α (ERα) and β (ERβ) estrogen receptors was determined by ligand competition binding assay. Ischemia/reperfusion injury was induced by temporary middle cerebral artery occlusion (MCAO). E2 or ZYC3 (100 μg/kg) was administered 2 hours or immediately before MCAO, respectively. Infarct volume was determined by 2,3,5-triphenyltetrazolium chloride staining. Cerebral blood flow was recorded during and within 30 minutes after MCAO by a hydrogen clearance method.

Results—ZYC3 significantly decreased toxicity of glutamate with a potency 10-fold that of E2. ZYC3 did not bind to either ERα or ERβ. Infarct volume was significantly reduced to 122.4±17.6 and 83.1±19.3 mm^3 in E2 and ZYC3 groups, respectively, compared with 252.6±15.6 mm^3 in the ovariectomized group. During MCAO, both E2 and ZYC3 significantly increased cerebral blood flow in the nonischemic side, while no significant differences were found in the ischemic side. However, E2 and ZYC3 significantly increased cerebral blood flow in both sides within 30 minutes after reperfusion.

Conclusions—Our study shows that ZYC3, a non–receptor-binding estrogen analogue, possesses both neuroprotective and vasoactive effects, which offers the possibility of clinical application for stroke without the side effects of estrogens. It also suggests that both the neuroprotective and vasoactive effects of estrogen are receptor independent. **(*Stroke*. 2002; 33:2485-2491.)**

Key Words: cerebral blood flow ■ estrogens ■ ischemia ■ neuroprotection ■ receptors, estrogen ■ reperfusion injury

Neuroprotective agents and strategies have been studied for years and appear to be effective in a variety of stroke models. One of the major focuses in the last decade has been related to the activities of estrogens. Sex differences in the incidence and outcome of stroke suggest that hormonal factors may influence the development and outcome of stroke.[1,2] Estrogens have been found to be associated with a decreased risk, delayed onset, and progression of stroke and enhanced recovery from numerous traumatic and chronic neurological and mental diseases.[3] Various lines of clinical and experimental evidence have shown that both endogenous and exogenous estrogens exert neuroprotective effects.[3,4] The protective effects of estrogens have been widely reported in different types of neuronal cells against various toxicities, including serum deprivation, oxidative stress, amyloid β peptide, and excitotoxicity.[4] Furthermore, estrogens can decrease ischemia/reperfusion injury in animal studies.[5,6]

Although estrogens have been shown to be neuroprotective in both female and male rodent stroke models,[7] the feminizing effects limit their clinical application. Clinical trials have

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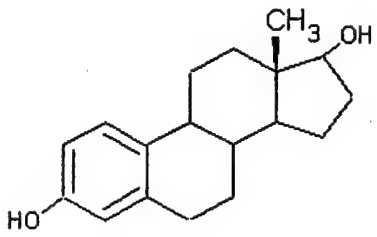
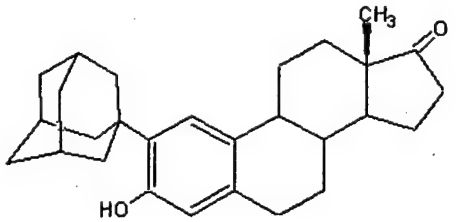


1,3,5(10)-estratrien-3, 17 beta-diol



2-adamantyl-estra-1,3,5(10)-trien-3-ol-17-one

Figure 1. Structural representation of 17β-estradiol and the novel estrogen analogue 2-adamantyl-estra-1,3,5(10)-trien-3-ol-17-one (ZYC3).

been limited to postmenopausal women because of problems in separating the cerebrovascular effects of estrogens from estrogenic effects on the peripheral estrogen-responsive tissues. In addition, both postmenopausal hormone replacement therapy and oral contraceptives have been found to increase the risk for venous and arterial thrombosis,[8,9] which has been shown to be related to the adverse events of the Heart and Estrogen-progestin Replacement Study (HERS).[10] The prothrombotic effect of estrogens may contribute to the negative results found in the HERS and the Women's Estrogen for Stroke Trial.[10,11] Many biological effects of estrogens are mediated by classic estrogen receptors (ERs), while ER-independent neuroprotection has been described.[4] Therefore, non–receptor-binding estrogen analogues, which preserve the neuroprotective effects but lack estrogenic activity in the peripheral estrogen-responsive tissue, could be applied in both females and males. In the present study a new derivative of estrone, 2-adamantyl-estra-1,3,5(10)-trien-3-ol-17-one (ZYC3), was synthesized (Figure 1), evaluated for ER binding, and assessed for its neuroprotective effects in both a neuronal cell culture model and in a rodent transient focal ischemia model. Because estrogens have been shown to be a vasorelaxant, we assessed the effects of this novel estrogen analogue on regional cerebral blood flow (CBF).

Materials and Methods

Synthesis of ZYC3 and Preparation of Steroid

ZYC3 was prepared as described previously.[12] Estrone (270 mg, 1 mmol) and 1-adamantanol (170 mg, 1 mmol) were added to anhydrous *n*-pentane (6 mL), and the mixture was cooled with an ice bath. Boron trifluoride etherate ($BF_3 \cdot EtOEt$, 0.4 mL) was added over a 10-minute period. After an additional 15 minutes, the ice bath was removed, and stirring was continued for an additional 45 minutes at room temperature. During the 45 minutes, the solid present in the reaction mixture was dissolved, and yellow oil formed. Crushed ice was then added while the reaction flask was shaken and swirled, and pink solid was formed. The filtered crude product was washed with water until the filtrate had a neutral pH, and the solid was dried in a vacuum oven at 50°C. The crude powder was purified by flash chromatography from contamination of other steroids (silica gel eluted with 20% ethyl acetate in hexanes). The product was recrystallized from a mixture of chloroform and isopropyl alcohol and had the following: melting point 322°C to 324°C, literature melting point 295°C to 296°C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.91 (s, 3H, C_{18}-CH_3), 2.8 (m, 2H, C_6-CH_2), 4.71 (s, 1H, C_3-OH), 6.42 (s, 1H, aromatic H), 7.15 (s, 1H, aromatic H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 13.76, 21.47, 25.93, 26.42, 28.63, 28.95 (3×C), 31.56, 35.81, 36.56, 36.98 (3×C), 38.42, 40.70 (3×C), 44.25, 48.00, 50.35, 116.87, 124.11, 131.59, 134.00, 135.02, 152.44, 221.43.

Steroids (Steraloids, Inc) were dissolved in absolute ethanol and then diluted to appropriate concentrations in culture media or assay buffer for cell culture or receptor-binding assays, respectively. For subcutaneous injection, 17β-estradiol (E2) and ZYC3 were dissolved in absolute ethanol and then dissolved in corn oil at a concentration of 100 μg/mL. To achieve a formulation that is aqueous soluble and therefore suitable for intravenous administration, ZYC3 was dissolved in aqueous 30% 2-hydroxypropyl-β-cyclodextrin (HPβCD) solution at a concentration of 100 μg/mL.

Ligand Competition Assay of ER Binding

Fifty microliters of 2,4,6,7-^{3}H-βE2 (NEN Life Science Products, Inc) and 100 μL of recombinant human ERα or ERβ (Affinity Bioreagents, Inc) were incubated in ER binding buffer (20 mmol/L Tris, 1 mmol/L EDTA, 400 mmol/L KCl, 1 mmol/L dithiothreitol, 10% glycerol, pH 7.8) for 75 minutes at 29°C either with no added steroid (total binding), 2 μL 1 mmol/L diethylstilbestrol (nonspecific binding; Steraloids), or 50 μL 0.001 to 10 nmol/L βE2 or ZYC3. Bound and unbound radioligands were separated with the use of Sephadex G25 (Amersham Pharmacia Biotech) columns (0.5-mL bed volume) with a 1-mL elution volume. Scintillation fluid was added, and counts were determined.

Effects of E2 and ZYC3 on Glutamate Toxicity in HT-22 Cells

HT-22 cells (Salk Institute) were maintained in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% charcoal-stripped fetal bovine serum (HyClone) and 20 μg/mL gentamycin under standard cell culture conditions. HT-22 cells (passages 18 to 25) were seeded into 96-well plates at a density of 5000 cells per well. E2 or ZYC3 was added at concentrations ranging from 1 nmol/L to 10 μmol/L immediately before the addition of glutamate. Glutamate was diluted to a final concentration of 10 mmol/L in culture media.

Cells were exposed to steroid and glutamate for approximately 14 hours, then cell viability was determined by calcein AM assay (Molecular Probes). Wells were rinsed with PBS, after which 25 μmol/L calcein AM in PBS was added. After incubation at room temperature for 15 minutes, fluorescence was determined (excitation 485, emission 530). All data were normalized to percent kill.

Experimental Animals

Female Sprague-Dawley rats (weight, 250 g; Charles River, Wilmington, Mass) were acclimatized for 3 days before surgery. Bilateral ovariectomy was performed 2 weeks before middle cerebral artery occlusion (MCAO). All animal procedures were approved by the University of North Texas Health Science Center Animal Care and Use Committee.

Ischemic stroke was induced by MCAO as described before.[13] Briefly, animals were anesthetized by intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). Rectal temperature was maintained at 37.5±0.5°C during the procedure. The left femoral artery was canalized and connected to a blood pressure monitor for mean arterial blood pressure monitoring. The left MCA was occluded by a 3-0 monofilament suture introduced via the

internal carotid artery. After 1 hour, the suture was withdrawn for reperfusion. Blood samples were taken before, 30 minutes during, and 30 minutes after MCAO. Physiological parameters were measured by an ISTAT clinical analyzer.

Animals were decapitated 24 hours after reperfusion. Brains were harvested, and 7 slices were made at 3, 5, 7, 9, 11, 13, and 15 mm posterior to the olfactory bulb. Slices were incubated for 30 minutes in a 2% solution of 2,3,5-triphenyltetrazolium chloride at 37°C and then fixed in 10% formalin. The stained slices were photographed and subsequently measured for ischemic lesion volume (Image-Pro Plus 4.1, Media Cybernetics).

A hydrogen clearance blood flowmeter (Unique Medical Co) was used for CBF measurement. Two electrodes were stereotaxically inserted into caudate putamen of the ischemic and contralateral side (posterior bregma 0.5 mm, 4 mm lateral and 5 mm depth).

Protocol 1: Effect of ZYC3 on Ischemia/Reperfusion Injury Using Corn Oil as Vehicle

E2 and ZYC3, dissolved in corn oil, were injected subcutaneously (100 μg/kg) 2 hours before MCAO in each group, respectively. An equivalent dose of vehicle was administered subcutaneously 2 hours before MCAO in ovariectomized females as control (n=4 to 6 per group).

Protocol 2: Effect of ZYC3 on Ischemia/Reperfusion Injury Using 30% HPβCD as Vehicle

ZYC3 (100 μg/kg) in HPβCD was administered through the jugular vein immediately before MCAO (ZYC3 group; n=7). E2 (100 μg/kg) in corn oil was injected subcutaneously in ovariectomized females 2 hours before MCAO (E2 group; n=15). In controls, ovariectomized females (OVX group; n=14) were treated with equivalent volumes of corn oil (subcutaneously) at 2 hours before and 30% HPβCD (intravenously) immediately before MCAO.

Protocol 3: Effect of E2 and ZYC3 on CBF During Ischemia/Reperfusion Injury

MCAO was induced in 16 rats (OVX, n=5; ZYC3, n=6; E2, n=5) in a separate study. Mean arterial blood pressure, pH, Po_2, Pco_2, Na^+, K^+, hemoglobin, HCO_3, So_2, and total carbon dioxide (Tco_2) were monitored before, 30 minutes during, and 30 minutes after MCAO. E2 was dissolved in corn oil and injected subcutaneously (100 μg/kg) 2 hours before MCAO in the E2 group. In the ZYC3 group, ZYC3 (100 μg/kg) in HPβCD was administered through the jugular vein immediately before MCAO. CBF was recorded at 30 minutes during MCAO and at 5, 15, and 30 minutes after reperfusion.

Statistical Analysis

All data are presented as mean±SEM. Cell death, ischemic volumes, and physiological parameters in each group were compared by 1-way ANOVA followed by Tukey tests. For CBF we used a linear mixed model (SAS PROC mixed; SAS Institute) to evaluate mean differences in treatment for each side of the brain.[14,15] The effects of treatment, time, and treatment by time interaction were considered fixed, while the response of animals was treated as random. This model took into consideration repeated-measures data (multiple CBF measurements were made for each rat), the inhomogeneous variance of CBF at 4 different time points, and the variation among rats. Linear contrast tests were used to distinguish treatment groups at specific time points. The difference for each comparison was considered significant at the $P<0.05$ level.

Results

Ligand Competition for Estrogen Receptor Binding

In competition binding experiments, ZYC3 showed very low affinity for both ERα and ERβ within the indicated concentrations (Figure 2).

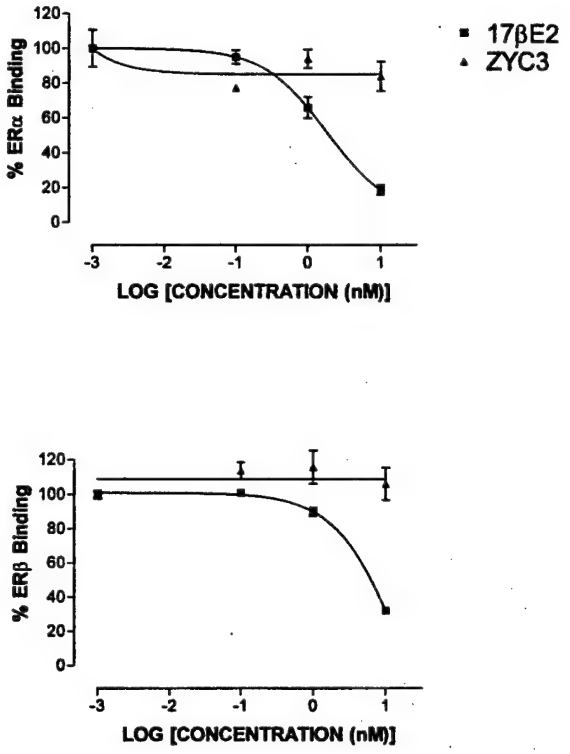


Figure 2. Competition binding curve of ZYC3 and E2 for human recombinant ERα and human recombinant ERβ. No binding affinity of ZYC3 to either ERα or ERβ was indicated.

Effect of E2 and ZYC3 on Glutamate Toxicity in HT-22 Cells

The EC_{50} values for neuroprotection against a 10-mmol/L glutamate challenge for ZYC3 and E2 were 0.16 and 1.90 μmol/L, respectively, suggesting that ZYC3 was 10-fold more potent than E2. With exposure of HT-22 cells to 10 mmol/L glutamate for 14 hours, 1 μmol/L ZYC3 ameliorated glutamate toxicity by 70%, while 1 μmol/L E2 decreased cell death by 10% (Figure 3).

Effect of E2 and ZYC3 on Ischemic Lesion Volume

ZYC3 was not neuroprotective when injected subcutaneously 2 hours before MCAO with corn oil used as vehicle. The lesion volumes were 238.1±23.9, 128.5±24.3, and 274.2±31.4 mm^3 in OVX, E2, and ZYC3 groups, respectively (Figure 4).

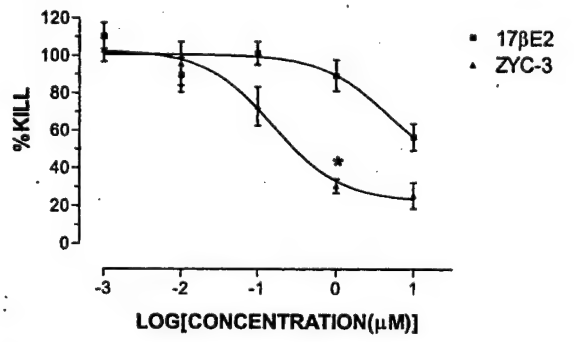


Figure 3. Concentration-response curves for E2 and ZYC3 on glutamate toxicity in HT-22 cells. *$P<0.05$ vs 17βE2 (E2) group.

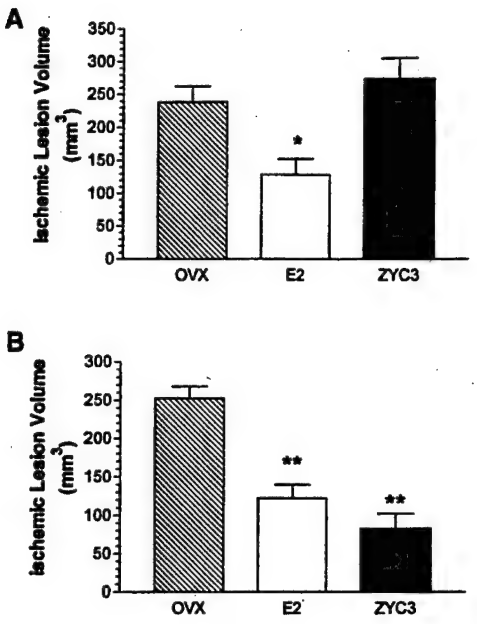


Figure 4. A, Effect of E2 and ZYC3 (100 μg/kg), dissolved in corn oil, on ischemic lesion volume of MCAO. *$P<0.05$ vs OVX and ZYC3 groups. B, Effect of ZYC3 (100 μg/kg) in 30% HPβCD and E2 on ischemic lesion volume of MCAO. **$P<0.05$ vs OVX group.

In view of the potent neuroprotection of ZYC3 seen in vitro and its high lipophilicity, we considered the possibility that the lack of in vivo neuroprotection with subcutaneous administration in oil may have been due to the lack of distribution of the compound from the injection site. To test this possibility, we complexed ZYC3 in HPβCD, creating a formulation that allowed aqueous solubility and rapid distribution through intravenous administration. The lesion volume was significantly reduced in the ZYC3 group when ZYC3 was administered intravenously in 30% HPβCD immediately before MCAO. The lesion volumes were 83.1±19.1, 122.4±17.6, and 252.6±15.6 mm^3 in ZYC3, E2, and OVX groups, respectively (Figure 4)

Effect of E2 and ZYC3 on Physiological Parameters and CBF

Physiological parameters are shown in the Table. There were no significant differences among groups at comparable experimental time points. Regional CBF decreased to 8.5±1.3, 6.3±1.4, and 7.4±1.4 mL/min per 100 g tissue during MCAO in the ischemic side in the ZYC3, E2, and OVX groups, respectively. However, both E2 and ZYC3 significantly increased CBF in the nonischemic side during MCAO, which was 91.6±10.7 and 82.7±11.7 mL/min per 100 g tissue, respectively, compared with 48.0±11.7 mL/min per 100 g tissue in the OVX group. E2 and ZYC3 significantly increased CBF in both sides within 30 minutes after reperfusion (Figure 5).

Discussion

A large number of reports have shown that E2, naturally occurring estrogen, exerts neuroprotective effects in a variety of in vitro and in vivo model systems.[5,16–20] Several synthesized estrogen analogues have also been reported to possess neuroprotective properties.[13,16,18,21] The most essential structural motif that elicits estrogenic activity is a phenol that is relatively unhindered, attached to a rather bulky hydrophobic structure.[22] Additionally, the neuroprotective function of estrogens has been shown to be related to the phenolic A ring of the steroid.[23] Adamantyl modulation of phenol has been reported previously.[24] In the present study an electron-donating, adamantyl moiety was introduced at the C-2 position on the A ring of the steroid. We anticipated that the bulky adamantyl group would decrease binding of the steroid to ERs. It was also anticipated that the bulky group would increase the ability of the steroid to scavenge free radicals derived from the reactive oxygen species. The effects of this novel analogue on ischemia/reperfusion injury were determined in both a neuronal cell culture model and a rodent MCAO model.

Oxidative stress is implicated in a number of neurological disorders, including stroke. HT-22 is an immortalized mouse hippocampal neuronal cell line, which lacks inotropic gluta-

Physiological Parameters Before, During, and After MCAO

Group	MABP, mm Hg	PH	Pco_2, mm Hg	Po_2, mm Hg	HCO_3, mm Hg	So_2, %	Na^+, mmol/L	K^+, mmol/L	Tco_2, mmol/L	Hb, g/dL
OVX (n=5)										
Before	77±1	7.32±0.04	51±4	67±6	26.0±0.6	89.2±3.0	140±1	5.02±0.19	27.6±0.4	14.0±0.6
During	83±2	7.33±0.01	48±1	69±4	26.0±0.4	91.0±1.8	140±1	4.82±0.21	27.0±0.3	14.0±0.3
After	83±3	7.30±0.02	49±2	72±6	24.8±0.6	91.0±2.4	141±1	4.64±0.23	26.4±0.4	14.2±0.4
ZYC3 (n=6)										
Before	80±5	7.33±0.02	52±2	72±4	27.0±0.5	92.0±1.2	139±1	4.85±0.11	28.0±0.9	15.0±0.3
During	78±2	7.34±0.01	47±2	75±5	25.7±0.6	93.0±1.4	140±2	4.55±0.21	26.8±0.7	14.8±0.2
After	80±5	7.35±0.01	44±1	77±6	24.8±0.2	93.7±1.6	142±1	4.28±0.26	26.8±0.5	15.0±0.4
E2 (n=5)										
Before	86±4	7.30±0.02	53±3	66±4	27.2±0.8	89.6±1.6	140±1	4.88±0.19	28.8±0.8	14.4±0.5
During	81±1	7.31±0.01	52±2	68±4	26.4±0.7	90.4±1.9	140±1	4.48±0.42	28.2±0.9	14.4±0.4
After	85±7	7.35±0.02	45±2	74±8	24.6±1.0	92.2±2.4	139±1	4.66±0.42	26.2±1.0	14.2±0.5

Hb indicates hemoglobin. There were no significant differences between treatment groups and the control OVX group.

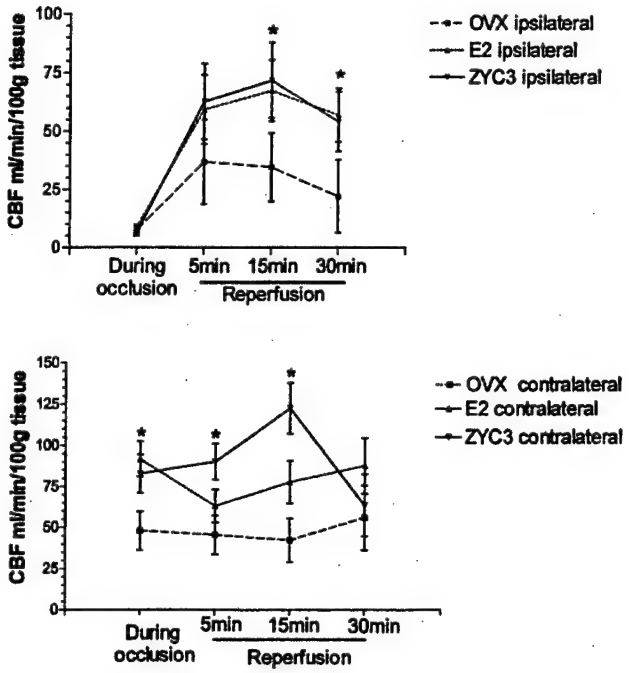


Figure 5. Effects of E2 and ZYC3 on CBF. Both E2 and ZYC3 significantly increased CBF in the nonischemic side during MCAO. E2 and ZYC3 significantly increased CBF in both sides within 30 minutes after reperfusion. *$P<0.05$ vs OVX group.

mate receptors.[25] HT-22 cells have been used as a model of oxidative toxicity on exposure to glutamate.[26] Exogenous glutamate blocks cysteine uptake in HT-22 cells via inhibition of the glutamate-cysteine antiporter, resulting in decreases in intracellular cysteine, which is 1 of the 3 peptides of glutathione. Depletion of glutathione leads to accumulation of reactive oxygen species and Ca^{2+} influx, ultimately resulting in cell death. The present study showed that both E2 and ZYC3 treatment ameliorated glutamate toxicity to HT-22 cells. With the free radical–scavenging adamantyl group, ZYC3 was more potent and efficacious than E2. Furthermore, because ZYC3 had very low affinity to either of the 2 known ERs and HT-22 cells lack functional ERs,[27] classic ER-independent neuroprotective actions of E2 were suggested.

Consistent with our in vitro studies, both E2 and ZYC3 exert neuroprotective effects in focal cerebral ischemia/reperfusion injury. For the in vivo study, ZYC3 was ineffective in protecting against the insult when administered in corn oil. The logarithm of the 1-octanol/water partition coefficient (log P) estimated by an atom fragment method[28] indicated that ZYC3 has a log P of 6.83 compared with 4.01 for E2. The extremely high lipophilicity indicated that ZYC3 should essentially accumulate in a lipid environment and would therefore remain in the injection site. The lack of effects of ZYC3 could be attributed to the inappropriate formulation method. To achieve a rapid delivery of ZYC3 into the circulation, ZYC3 was complexed with HPβCD. With this formulation, neuroprotective and vasoactive effects were clearly manifested.

Neuroprotective effects of E2 have been indicated at a very large range of concentrations in both in vitro and in vivo studies. In in vitro studies, the effective concentrations for E2-mediated neuroprotection range from low nanomolar (approximately 0.1 nmol/L) to high micromolar (approximately 50 μmol/L) concentrations.[4] High physiological concentrations (low nmol/L) were sufficient to attenuate toxicity in a variety of cell types,[4] while significantly higher pharmacological concentrations (low μmol/L) were required to lessen glutamate toxicity in HT-22 cells. In in vivo studies, neuroprotective properties of E2 have been observed in both low physiological and high pharmacological concentrations. A myriad of factors contribute to the vast differences in neuroprotection of E2 concentrations in vitro. In cell culture studies, differences in cell type, culturing conditions, cell density, and media components may contribute to the wide range of protective concentrations. Other factors that may also alter the potency of E2 neuroprotection include type of insult, severity of insult, and time of beginning of treatment. In the present study 10 mmol/L glutamate was used as insult. The neuroprotective potency of E2 was 1.9 μmol/L, which is consistent with previous studies.[13]

Many actions of estrogens are mediated by the binding of the steroid to the nuclear ERs, and the binding of the steroid-receptor complex to the ER response element thereby activates transcriptional events. However, it has become evident that estrogens exert activities in neurons independent of the activation of the classic ERs.[27,29,30] Several lines of evidence suggest that the neuroprotective effects of estrogens do not require ER-dependent gene transcription. First, several non–receptor-binding estrogen analogues such as the enantiomer of E2 have been shown to exert neuroprotective efficacy as potent as E2.[13,16,18,31] Second, ER antagonists do not attenuate the protective action of E2 in all models of neurotoxicity.[21,32] Third, neuroprotection of estrogens can occur in the presence of mRNA or protein synthesis inhibitors.[31,33] Furthermore, E2 has been shown to activate a signal transduction pathway involving mitogen-activated protein kinase within minutes of E2 treatment, which is too rapid to involve genomic ER actions.[34,35] Taken together, the present study suggests that many of the neuroprotective effects of estrogens are mediated through nongenomic pathways independent of classic ERs.

Vasoactive effects of estrogens in the central nervous system have been suggested. Estrogens can increase CBF during global ischemia and restore postischemic pial microvascular dilation.[36,37] Estrogens can increase CBF during early reperfusion in an ischemia/reperfusion model.[38] In the present study rapid vascular responses were observed in both E2 and ZYC3 treatment groups. Our study indicated that vasoactive effects of estrogens were not through the classic genomic pathway, which is consistent with previous studies.[39–41] Because the vascular response of estrogen was determined in the core area of the ischemia, which was not protected by either E2 or ZYC3, the association between neuroprotective properties and vasoactive activity of estrogens cannot be determined in the present study.

Although classic ER-independent neuroprotective effects are suggested by the present study, evidence for ER-dependent action has also been reported. Estrogens have been shown to activate the phosphatidylinositol-3-kinase pathway in vitro, which has been proposed to mediate inhibition of

apoptosis and support neuronal survival. This activation can be inhibited by ER antagonists.[42,43] However, whether this pathway is necessary and sufficient to mediate the neuroprotective effects of estrogens in vivo still remains to be elucidated. It has also been suggested that ERα, but not ERβ, was critical for the neuroprotective properties of estrogen in one ERα and ERβ knockout (αERKO, βERKO) study,[44] while another study did not support this conclusion.[45] The differences between the ERKO studies could have resulted from the different estrogen concentrations used in the 2 treatment regimens or from the different MCAO models, permanent versus transient, used in these 2 studies. Higher doses of E2 have been demonstrated to exert neuroprotective effects in αERKO mice.[46] These higher doses of E2 could be the result of the elevated levels of androgen in αERKO mice,[47] since androgen has been shown to exacerbate ischemia/reperfusion injury.[48]

In summary, our in vitro and in vivo study suggests that this novel estrogen analogue exerts both neuroprotective and vasoactive effects. The low affinity of binding of ZYC3 to the classic ERs and rapid cerebrovascular action of this estrogen derivative indicate that the neuroprotective and vasoactive effects of estrogens are mediated through nongenomic pathways independent of classic ERs. This non–ER-binding estrogen analogue could be applied in situations in which the feminizing effects of estrogens are undesirable and to avoid other receptor-dependent side effects of estrogens.

Acknowledgments

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Appendix D

Estradiol Exerts Neuroprotective Effects When Administered After Ischemic Insult

Shao-Hua Yang, MD; Jiong Shi, PhD; Arthur L. Day, MD; James W. Simpkins, PhD

Background and Purpose—17β-Estradiol (E2) has been reported to exert neuroprotective effects when administered before an ischemic insult. This study was designed to determine whether E2 treatment after ischemia exerts the same effects and, if so, how long this therapeutic window remains open, and whether the effects are related to changes in cerebral blood flow (CBF).

Methods—Female Sprague-Dawley rats were subjected to permanent middle cerebral artery occlusion (MCAO). In protocol 1, E2 was administered (100 μg/kg IV followed immediately by subcutaneous implantation of crystalline E2 in a silicone elastomer tube) to ovariectomized females (OVX+E2) at 0.5 (n=8), 1 (n=6), 2 (n=7), 3 (n=6), or 4 (n=9) hours after MCAO. Intact (INT; n=6) and ovariectomized females (OVX; n=12) were subjected to MCAO and received vehicle instead of E2. Two days after MCAO the animals were killed, and ischemic lesion volume was determined by 2,3,5-triphenyltetrazolium chloride staining. In protocol 2, CBF was monitored before and at 1, 24, and 48 hours in a group of animals receiving E2 or vehicle 0.5 hour after ischemia induction (INT, n=6; OVX, n=8; OVX+E2, n=6).

Results—Lesion volume was 20.9±2.2% and 21.8±1.2% in the INT and OVX groups, respectively. E2 was found to decrease lesion volume significantly when administered within 3 hours after MCAO. The lesion volumes were 6.3±0.5%, 10.3±2.1%, 11.8±1.8%, 13.5±1.6%, and 17.9±2.8% when E2 was administered at 0.5, 1, 2, 3, or 4 hours after MCAO, respectively. CBF decreased to 43.1±2.2% and 25.4±1.0% in the INT and OVX animals, respectively, at 5 minutes after MCAO. In comparison to OVX rats, CBF was not different at 1 hour after E2 administration but was increased significantly in the OVX+E2 group 1 and 2 days after E2 administration.

Conclusions—E2 exerts neuroprotective effects when administered after ischemia, with a therapeutic window in a permanent focal cerebral ischemia model of approximately 3 hours. This effect of estradiol was associated with no immediate change in blood flow but with a delayed increase in CBF. **(*Stroke*. 2000;31:745-750.)**

Key Words: cerebral blood flow ■ estrogens ■ ischemia ■ neuroprotection

Both retrospective and prospective epidemiological studies have demonstrated beneficial effects of estrogen replacement therapy in reducing stroke-related mortality that is associated with stroke in postmenopausal women.[1,2] Recently, several laboratory studies have also emphasized the neuroprotective effects of estrogens.[3–6] Both chronic and acute pretreatment can reduce ischemic damage in focal cerebral ischemia, indicating that estrogens may be a new therapeutic class of drugs to prevent neuronal damage associated with cerebral ischemia.

Presently, it is not known whether postischemic treatment with estrogen is beneficial. The purpose of this study was to determine (1) whether 17β-estradiol (E2) can protect against brain injury when administered after cerebral ischemia; (2) the duration of any therapeutic window offered by E2, and (3) whether any E2 neuroprotective effects are associated with changes in cerebral blood flow (CBF).

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Materials and Methods

Female Charles River Sprague-Dawley rats (225 to 250 g; Wilmington, Mass) were maintained in laboratory acclimatization for 3 days before ovariectomy. Bilateral ovariectomy was performed 2 weeks before middle cerebral artery occlusion (MCAO) under methoxyflurane inhalant anesthesia. All animal procedures were approved by the University of Florida Animal Care and Use Committee.

Middle Cerebral Artery Occlusion

Animals were anesthetized by intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). Rectal temperature was monitored and maintained between 36.5°C and 37.5°C during the procedure. MCAO was achieved according to the methods described by others, with the following modifications.[7,8] With the aid of an operating microscope, the left common carotid artery, external carotid artery and internal carotid artery were exposed through a midline cervical skin incision. A 4-0 monofilament suture with its tip

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rounded by heating was introduced into the internal carotid artery via the external carotid artery lumen and advanced until resistance was encountered. The distance between the common carotid artery bifurcation and the resistive point was approximately 1.9 cm. A 6-0 silk ligature was placed around the external carotid artery and tightened around the intraluminal monofilament suture to prevent bleeding and change of the suture position. The common carotid artery and pterygopalatine artery temporary ligatures were then released, and the skin incision was closed.

Measurement of Regional CBF

A laser-Doppler flowmeter was used for CBF measurements. The scalp was incised on the midline, and bilateral 2-mm burr holes were drilled 1.5 mm posterior and 4.0 mm lateral to the bregma. The dura was left intact to prevent cerebrospinal fluid leakage. Laser-Doppler flowmeter probes held in place by a micromanipulator were stereotaxically advanced to gently touch the intact dura mater. CBF was measured before and within 1.5 hours after MCAO. The incision was stapled, and the animals were then returned to their home cages. At 1 and 2 days after MCAO, the animals were reanesthetized with ketamine (60 mg/kg IP) and xylazine (10 mg/kg IP), and stable CBF recordings were obtained bilaterally at the same sites for at least 10 minutes. The CBF values were calculated and expressed as a percentage of the baseline values. CBF values reported represent the mean±SEM for the average of the CBF recordings obtained.

Measurement of Lesion Volume

Each group of animals was decapitated 2 days after MCAO, and the brain was removed and placed in a metallic brain matrix for tissue slicing (Harvard) immediately after decapitation. Five slices were made at 3, 5, 7, 9, and 11 mm posterior to the olfactory bulb. Each slice was incubated for 30 minutes in a 2% solution of 2,3,5-triphenyltetrazolium chloride in physiological saline at 37°C and then fixed in 10% formalin. The stained slices were photographed by a digital camera (Sony MVC-FD5) and subsequently measured for the surface area of the slices and the ischemic lesion (Image-Pro Plus 3.0.1). Ischemic lesion volume was calculated as the sum of the areas of the ischemic lesion across the 5 slices divided by the total cross-sectional area of these 5 brain slices.

E2 Administration and Serum Concentration

To obtain a prompt and sustained elevation in serum E2 concentration, intravenous injection of an aqueous soluble E2 preparation combined with simultaneous implantation of a silicone elastomer pellet containing the steroid was used. To assess serum concentrations of E2 after this treatment regimen, 6 OVX animals were anesthetized with methoxyflurane inhalant, and a control blood sample was taken via the jugular vein. Then E2 (100 μg E2/kg body wt) complexed with hydroxypropyl-β-cyclodextrin (E2-HPCD, Sigma), which was dissolved in 0.9% normal saline, was administered via tail vein injection, and a 5-mm-long silicone elastomer tube (1.57 mm ID; 3.18 mm OD) containing crystalline E2 was immediately implanted subcutaneously. The animals were put back into their cages, and blood samples were then taken via the jugular vein at 5 minutes and 0.5, 1, 2, 4, 6, 12, 24, and 48 hours after steroid administration, under methoxyflurane inhalant anesthesia. Serum was separated from blood by centrifugation and stored frozen (−20°C). Serum E2 concentrations were determined with the use of duplicate serum aliquots in a radioimmunoassay (ultrasensitive estradiol kit, Diagnostic Laboratory).

Protocol 1

To determine whether E2 exerts any beneficial neuroprotective effect when administered after the ischemic insult and the duration of any therapeutic window, E2 was administered (100 μg/kg, by tail vein injection combined immediate with subcutaneous implantation of an E2-containing silicone elastomer tube) in ovariectomized female rats (OVX+E2 group) at 0.5 (n=8), 1 (n=6), 2 (n=7), 3 (n=6), or 4 (n=9) hours after MCAO. As controls, ovariectomized females (OVX group; n=12) and intact females (INT group; n=6) were treated with equivalent volumes of saline and empty pellets at 0.5, 1, 2, 3, or 4 hours after MCAO.

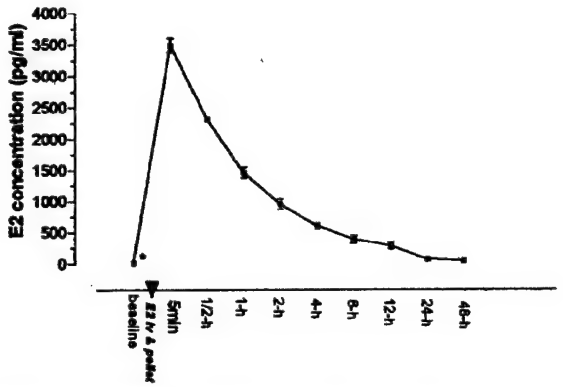


Figure 1. Effect of simultaneous intravenous injection of E2 (100 μg/kg complexed in hydroxypropyl-β-cyclodextrin) and subcutaneous implantation of an estradiol-filled silicone elastomer pellet on serum estradiol concentration in OVX rats. At the time indicated, 0.5-mL blood samples were obtained for subsequent E2 concentration analysis. n=6 rats per time point. Mean±SEM values are depicted. *$P<0.05$ vs all other sample times.

Protocol 2

To determine whether any neuroprotective effects of E2 were associated with blood flow changes, a laser-Doppler flowmeter was used to monitor CBF. After a baseline CBF reading was obtained, CBF was continuously recorded for 1.5 hours after MCAO induction. E2 was administered (100 μg/kg tail vein injection and subcutaneous implantation of an E2 pellet) 0.5 hour after MCAO induction (OVX+E2 group; n=6), and CBF was obtained for 1 hour thereafter and at 24 and 48 hours after MCAO. Intact females (INT group; n=6) and ovariectomized females (OVX group; n=8) received equivalent volumes of saline and empty pellet as controls.

Statistical Analysis

Statistical analyses were performed with SigmaStat 2.0 Software (Jandel Scientific). All data were expressed as mean±SEM. The lesion volumes in each group comparison were analyzed with 1-way ANOVA. The CBF values in each group were analyzed among groups at each sampling time with 1-way ANOVA and multiple comparisons. The difference for each comparison was considered significant at the $P<0.05$ level.

Results

Effects of E2 Administration on Serum E2 Concentration

In young cycling female rats, serum levels of E2 vary between 11±1 pg/mL at diestrus and 41±5 pg/mL at proestrus. Serum E2 concentrations increased and peaked at 3487±110 pg/mL 5 minutes after E2 administration, then decreased to 76±16 pg/mL 24 hours after administration (Figure 1). With the slow release from the E2 pellet, serum E2 concentration remained high at 45±5 pg/mL 48 hours after administration, compared with 13±4 pg/mL in OVX animals.

Therapeutic Window of E2

E2 treatment after the ischemic insult exerted neuroprotective effects (Figures 2 and 3). The ischemic lesion volume was significantly reduced in the OVX+E2 group when E2 was administered at 0.5, 1, 2, or 3 hours after the ischemic insult, with lesion volumes of 6.3±0.5%, 10.3±2.1%, 11.8±1.8%,

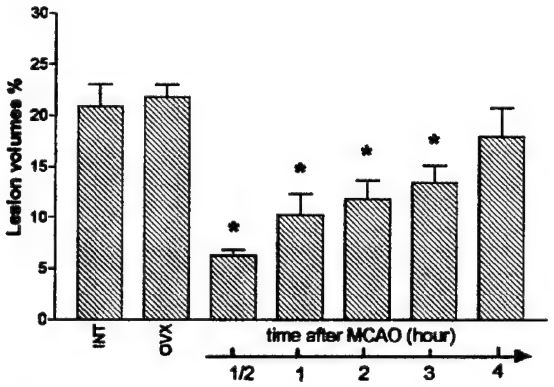


Figure 2. Effects of E2 treatment on lesion volume after MCAO in OVX and OVX+E2 rats. E2 was administered by simultaneous intravenous injection (100 μg/kg) and subcutaneous implantation of an E2 pellet at 0.5 (n=8), 1 (n=6), 2 (n=7), 3 (n=6), or 4 (n=9) hours after MCAO. Mean±SEM values are depicted. *$P<0.05$ vs OVX and INT.

and 13.5±1.6%, respectively ($P<0.05$), indicating a therapeutic window of up to 3 hours in permanent focal cerebral ischemia. No significant difference of lesion volume was noted between OVX and INT groups (21.8±1.2% and 20.9±2.2%, respectively).

Effect of E2 on CBF

The ipsilateral CBF was higher immediately after MCAO in the INT group compared with the OVX and OVX+E2 groups: values for INT, OVX, and OVX+E2 groups were 43.1±2.2%, 26.2±1.5%, and 23.9±0.9%, respectively ($P<0.01$). After E2 administration, ipsilateral CBF increased at 1 and 2 days after E2 administration but not at 1 hour (Figure 4). The effects of MCAO on the contralateral CBF were similar in all groups and were independent of the estrogen status of the animal.

Discussion

This study demonstrates 3 potentially important clinical effects of E2. First, E2 exerts neuroprotective effects even

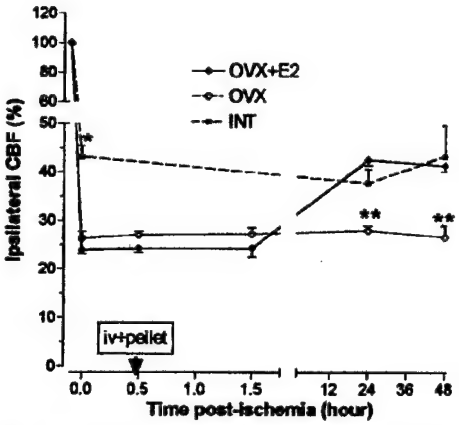
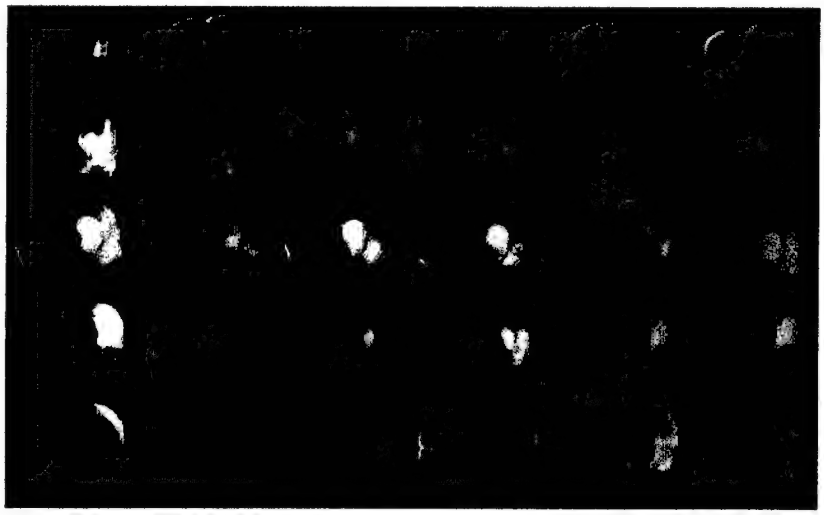


Figure 4. Effect of ovarian status and E2 replacement on ipsilateral CBF after MCAO. E2 was administrated by an intravenous injection (100 μg/kg) and subcutaneous implantation of an E2 pellet at 0.5 hour after MCAO. CBF was measured before and within 1.5 hours after MCAO (OVX, n=8; OVX+E2, n=6; and INT, n=6) and at 24 and 48 hours after MCAO. Mean±SEM values are depicted. *$P<0.05$ vs OVX and OVX+E2; **$P<0.05$ vs INT and OVX+E2.

when administered after the onset of an ischemic insult, with a therapeutic window up to 3 hours. Second, the neuroprotective effects of E2 are not associated with an immediate blood flow augmentation effect but with a later improvement in CBF. Third, at the dose used, neuroprotective effects of E2 are flow independent and in this permanent focal cerebral ischemia model are only observed with exogenous E2.

Several studies have demonstrated that E2 is a potent neuroprotective agent that decreases focal ischemia–induced lesion size by approximately 50% with E2 chronic pretreatment.[3–6] E2 also exerts neuroprotective effects when administered immediately before occlusion.[9] The present study, for the first time, systematically defines the therapeutic window of E2 in a model of permanent focal ischemia when the drug is administered after the ischemia has been induced.



OVX | OVX+E2 ½-hour | OVX+E2 1-hour | OVX+E2 2-hour | OVX+E2 3-hour | OVX+E2 4-hour

Figure 3. Photographic display of representative brain sections from OVX and OVX+E2 rats at different time points (0.5 hour, n=8; 1 hour, n=6; 2 hours, n=7; 3 hours, n=6; and 4 hours, n=9). Note the increasing infarct size with greater delays in drug administration.

The neuroprotective mechanisms of E2 are not yet elucidated, although both direct neuroprotective action on neurons and indirect effects on the cerebral vasculature are possible. Direct effects can include reduction in reactive oxygen species that accumulate during ischemia,[10] blockade of excitatory amino acid toxicity,[11,12] modulation of calcium homeostasis,[13–15] induction of neurotrophins and their receptor and intracellular signaling pathway,[16,17] induction of antiapoptotic protein,[18,19] and/or enhancement of brain glucose uptake.[20] E2 could also improve the outcome of cerebral ischemia through a protective effect on brain vascular endothelial cells,[20] resulting in the presently observed delayed improvement in CBF in E2-treated rats.

E2 has been shown to act on both of the peripheral and intracranial vascular systems.[21–24] In young cycling female rats, serum levels of E2 varied between 11±1 pg/mL on diestrus and 41±5 pg/mL on proestrus.[25] In our study, deprivation of endogenous ovarian steroids resulted in low residual CBF ipsilateral to the MCAO.[5,26] Acute administration of exogenous E2 (in which serum levels of E2 vary from 3487±110 to 45±5 pg/mL) increased ipsilateral CBF after stroke, but this effect was delayed until 1 to 2 days after occlusion. It appears that low levels of endogenous ovarian steroids resist the ipsilateral CBF effects after permanent MCAO. Acute treatment with high doses of E2 caused a delayed preserving effect on CBF, an effect that only occurred in the side ipsilateral to the MCAO.

The mechanism of any blood flow–preserving effects of E2 is still not well known, but 3 possibilities have been proposed. First, we have found that exposure of endothelial cells to E2 helps to maintain their viability during an ischemic episode.[20] Findings in this experiment suggest that the delayed effect of E2 on CBF maybe be secondary to a vascular cytoprotective action of the hormone. Alternatively, estrogen could induce vasodilation in cerebral arteries.

Second, E2 has been found to modulate serum lipid levels, reducing aggregation of platelets and the thrombotic and vasoconstrictive effects of thromboxane.[27,28] E2 withdrawal after ovariectomy increases the sensitivity of the rabbit basilar artery to serotonin.[29] Using a mouse carotid model, Sullivan et al[23] found that physiological levels of E2 replacement could significantly suppress the response of the carotid artery to injury. The endothelium produces a variety of vasoactive mediators such as prostacyclin and endothelium-derived nitric oxide, both of which have roles in regulating not only vascular tone but also smooth muscle cell proliferation.[21] Goldman et al[30] have also reported that within 10 minutes of injection of a supraphysiological dose of E2, CBF increases to most regions of the brain. In contrast, our study showed that the blood flow–preserving effects of E2 are not immediate but occur from 1 to 24 hours after E2 administration. These blood flow–preserving effects could be likely due to a slower genomic effect, since the cellular effects of E2 on gene expression occur hours to days after any insult.[31]

Finally, E2 could cause a delayed improvement in CBF through angiogenic mechanisms. Recently, Morales et al[32] found that E2 exerted angiogenic effects in peripheral vessels. While angiogenic effects of E2 may play a potential role in protecting against cerebral ischemia, we are not aware of studies demonstrating that estrogens can induce angiogenesis within 2 days of steroid replacement. However, by promoting neovascularization and collateral formation, E2 could restore cerebral perfusion in ischemic areas and hence lessen the impact of occlusion.

Both low and high circulating concentrations of E2 have been reported to exert neuroprotective effects in the temporary cerebral ischemia model in E2 pretreatment studies.[5,33] Both low and high physiological levels of E2 have exerted similar effects in a 1-day permanent cerebral ischemia study when administered before ischemia.[6] The present study showed that E2 neuroprotective effects could be induced by high-level exogenous E2 in 2-day permanent cerebral ischemia when administered after ischemia. Subsequent assessment of the dose dependence of this neuroprotection is clearly needed.

Assessments of efficacy also need to be conducted in both male and female rats. E2 has been found to exert neuroprotective effects in males, although in males the effects are dependent in part on the suppression of testosterone secretion.[34] Additionally, the neuroprotective effects of estrogens do not appear to be mediated by an estrogen receptor mechanism. 17α-Estradiol, a very weak estrogen, exerts neuroprotective effects equivalent to E2 both in vitro and in vivo.[3,35] Additionally, we have recently reported that ent-estradiol, the enantiomer of E2 that lacks estrogenic activity, is as potent as E2 in protecting cerebral tissue from MCAO.[36] These data indicate that several nonfeminizing estrogens that lack classic genomic-mediated estrogenic effects are potential clinical candidates for stroke neuroprotection.

In summary, our study demonstrates that E2 exerts neuroprotective effects when administered after an ischemic insult, with a therapeutic window of approximately 3 hours. The neuroprotective effect has a delayed CBF-preserving component and a blood flow–independent component. This study raises the possibility that estrogen compounds could be a useful therapy in preserving brain tissue, even if administered after the ischemic insult.

Acknowledgments

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Editorial Comment

Clinical studies have demonstrated that chronic estrogen use reduces stroke-related mortality.[1,2] Along similar lines, animal models of cerebral ischemia have demonstrated that the presence of estrogen in physiological amounts is protective.[3–8] Although much evidence exists that estrogen reduces stroke-related morbidity and mortality when present at the time of injury, it has been unclear whether estrogen is of therapeutic utility when administered after an ischemic event has occurred. To be of utility in the treatment, as opposed to the prevention, of stroke, estrogen must exert a protective effect when given within a reasonable time window after the ischemic event. The article by Yang et al demonstrates that postischemic administration of estrogen affords protection against ischemic damage similar to preischemic administration and that it acts within a clinically useful therapeutic window. However, this postischemic protection only occurs at supraphysiologic doses of estrogen. Another study[5] has suggested that preischemic administration of supraphysiologic doses of estrogen lacks the neuroprotective activity exhibited by physiological doses of estrogen. Differences between the mechanisms of action of physiological and pharmacological amounts of estrogen must be determined to account for the differing actions when estrogen is administered before or after the ischemic event. It remains to be seen whether the mechanism by which supraphysiologic doses of estrogen exert a protective effect is a novel one or represents a nonspecific action of estrogen at a previously described neuroprotective site.

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Appendix E

DOSE DEPENDENCE AND THERAPEUTIC WINDOW FOR THE NEUROPROTECTIVE EFFECTS OF 17 β-ESTRADIOL WHEN ADMINISTERED AFTER CEREBRAL ISCHEMIA

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Dose dependence and therapeutic window for the neuroprotective effects of 17 β-estradiol when administered after cerebral ischemia

This manuscript includes figures and an acknowledgement

Word count: 4434

Key Words: Estrogen; Cerebral-ischemia; Neuroprotection

Abstract

Although much evidence have demonstrated that 17β-estradiol exerts neuroprotective effect when present at the time of injury or administration after ischemic event occurred, it is unclear whether the neuroprotective effects of supraphysiologic doses of estrogen is dose-dependent and if higher does of estrogen can prolong the 3 hours post treatment window. The present study was undertaken to determine if the neuroprotective effect of 17β-estradiol when administrated after ischemia is dose-dependent, and if higher doses of estrogen can prolong the therapeutic window. Ischemia injury was induced by permanent middle cerebral artery occlusion (MCAO) for 48 hours. 17β-estradiol 100 μg/kg or 500 μg/kg subcutaneous injected at 30 minutes after the ischemia resulted in a 36% and 63% reduction in lesion volume, respectively. This estrogen-mediated protection was observed in both cortex and subcortex regions of the ischemic infarct. Furthermore when a higher does of 17β-estradiol (1mg/kg) was subcutaneous injected at 6 hour after the ischemia, 33% of the rats still showed a 57% reduction in lesion volume. These findings suggest that postischemic sc estrogen treatment with oil formulations affords protection against ischemic damage and the protection is dose-dependent and that it acts within a clinically useful therapeutic window.

Keywords: Estrogen; Cerebral-ischemia; Neuroprotection

Introduction.

We initially reported that estrogens cause a dose-dependent protection of SK-N-SH cells under conditions of serum- deprivation. [1-2]. Since these initial observations of *in vitro* neuroprotection with estrogen treatment, there have been more than 80 reports describing the neuroprotective effects of estrogens see [2-9]. This neuroprotection is seen in a variety of neuronal cell types in response to over 14 different neuotoxic insults *in vitro*, including serum-deprivation [1-3,10], β-amyloid [11-14] and excitatory amino acid treatment [13,15,16].

We have now extensively assessed the neuroprotective effects of estrogens in an animal model of cerebral ischemia. Following our first reports of neuroprotection with estrogens in an animal model of ischemia [17-19] we and other have demonstrated that estrogens protects the brain from ischemic damage induced by transient cerebral ischemia [20-24], permanent cerebral ischemia [25, 26], subarachnoid hemorrhage [26], and global ischemia [27]. The protective effects of estrogens are seen with 17β-estradiol, as well as non-feminizing estrogens, such as 17α-estradiol [17], ENT-estradiol [28], and 2-adamantyl-estrone [29] suggesting that at pharmacological doses, estrogen receptors are not required for neuroprotection in stroke. Recently, Dubal et al. [30] reported that ERαKO, but not ERβKO mice were resistant to the neuroprotective effects of 17 β-E2 administered chronically at low concentrations and concluded that ERα is a necessary mediator of estrogen neuroprotection. Later, however, McCullough et al [31] demonstrated estrogen neuroprotection in ERαKO mice using pharmacological doses of the estrogens.

While much is known about the ability of pretreatment with estrogens to protect brain tissue from ischemia, few studies have assessed the effects of administration of estrogens after the onset of ischemia. In an initial studies, we reported that the protection afforded by 17β-estradiol when high doses are administered by an intravenous injects can be observed between 30 min and 3 hours following the onset of cerebral ischemia [17]; 32]. However, the precise therapeutic window for protection with estrogens is not known nor is the dose-dependency of estrogen protection after the onset of a stroke. The present study addressed both these issues using a subcutaneous preparation for rapid, sustained delivery of estrogens to the body.

Material and Method

Experimental animals

Female Charles Rivers Sprague-Dawley rats (250g, Wilmington, MA) were acclimatized to animal facilities three days prior to surgery. Bilateral ovariectomy was performed, under methoxyflurane inhalant anesthesia, 2 weeks before permanent middle cerebral artery occlusion (MCAO). All animal procedures were approved by the University of Florida and the University of North Texas Health Science Center Animal Care and Use Committees.

E_2 administration

Steroid (E950, Steraloids, Inc. Wilton, NH) was dissolved in absolute ethanol and then in sesame oil (Penta Manufacturing Company, Livingston, NJ) and the ethanol was evaporated off. Estradiol was dissolved at a concentration of 100μg/ml, 500μg/ml,

1mg/ml and 2% benzyl alcohol (EM Science, Gibbstown, NJ) was added to the solution to yield the 1ml/kg injection volume. A single subcutaneous injection of 100μg/kg, 500μg/kg, or vehicle was given to rats at 30 min after MCAO. In a separate study, a higher dose of 17β-estradiol (1mg/kg) or its vehicle was injected at 30 min or 6 hours after MCAO.

E_2 serum concentrations

We determined serum E_2 concentrations and the time course for E_2 reduction produced in rats given these supraphysiologic doses of estradiol. A group of animals (n=7) were ovariectomized to eliminate endogenous ovarian steroids. After two weeks, 100 μg/kg (the lowest dose used in our study) 17β-estradiol was injected subcutaneously (s.c.) to the rats. Blood samples were taken via a permanent atrial cannula prior to administration of the compound (time 0), and 5, 15, 30 and 60 min (0.5 ml) and 4, 8, 24 hours (1.0 ml) after E_2 administration. Blood samples were centrifuged for 20 minutes and plasma was separated and stored at -20°C until assayed. Serum E2 concentrations were determined using duplicate serum aliquots with an ultra-sensitive estradiol radioimmunoassay kit (Diagnostic Systems Lab, Webster, TX, U.S.A.) according to the manufacturer's instructions. This assay is sensitive to 2pg/ml sample and linear through 750pg/ml. For samples that were too high for the standard curve, plasma was diluted in assay buffer and diluted samples were reassayed.

Cerebral ischemia

Two weeks after ovariectomy, animals were anesthetized by intraperitioneal (i.p.) injection of ketamine (60mg/kg) and xylazine (10 mg/kg). During the procedures, rectal

temperature was monitored and maintained between 36.5°C and 37°C with heating lamps and gel warming pads. The left middle cerebral artery was permanently occluded using previously described methods [32]. Briefly, with the aid of an operating microscope (Zeiss, Thornwood, NY), the left common carotid artery and internal carotid artery were exposed through a midline cervical skin incision. A 3.0-cm length of 4-0 monofilament suture with a rounded-tip was introduced into the internal carotid artery via the external carotid artery lumen and advanced until resistance was encountered. The distance between the common carotid artery bifurcation and the resistant point was 2.2 cm. A 6-0 silk ligature was placed around the external carotid artery to prevent bleeding and movement of suture position. The common carotid artery and pterygopalatine artery temporary ligatures were then released, and the skin incision was closed.

Measurement of Lesion Volume

Animals were decapitated 48 hours after MCAO, and the brain was harvested and placed in a metallic brain matrix for tissue slicing (Harvard Apparatus, Holliston, MA). Seven 2mm coronal slices were made at 3, 5, 7, 9, 11, 13, and 15 mm posterior to the olfactory bulb. Each slice was incubated for 30 minutes in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC, Sigma Chemical Corp, St. Louis, MO) followed by fixation in 10% formalin. Stained slices were photographed by a digital camera (Sony MVC-FD5, Tokyo, Japan) and subsequently measured for injured surface area of the slices and the ischemic lesion (Image-Pro Plus 4.1, Media Cybernetics, Silver Spring, MD).

Protocol 1

This study was conducted to determine if the neuroprotective effects of 17β-estradiol was dose-dependent when administrated 30 minutes after MCAO. A total of 17 rats were divided into 3 groups. The animals were treated either with a single subcutaneous injection of 100 μg/kg 17β-estradiol (OVX+ E_2 100 μg/kg group, n=5) or 500 μg/kg 17β-estradiol (OVX+E_2 500 μg/kg group, n=7). As controls ovariectomized females (OVX groups, n=7) were subcutaneous injected with equivalent volumes of the vehicle (sesame oil with 2% of benzyl alcohol). The animals were decapitated 2 days after MCAO and the ischemic lesion volumes were measured.

Protocol 2

Our previous study demonstrated that E_2 exerts neuroprotective effects for up to 3 hours when intravenously administrated at concentrations of 100 μg/kg after an ischemic insult [32]. Herein, we tested the possibility that higher doses of E_2 could prolong this therapeutic window.

To define the treatment window for 17β-estradiol at higher doses after MCAO, ovariectomized rats were treated with subcutaneous injection of either 1mg/kg 17β-estradiol or its vehicle 30 minutes or 6 hours after the onset of the MCAO. 17β-Estradiol was administered as a single dose of 1 mg/kg body weight at 30 minutes (OVX+E_2 30 minutes group, n=6) or 6 hours (OVX+E_2 6 hours group, n=12) after MCAO. Control animals received the vehicle (sesame oil with 2% benzyl alcohol) at 30 minutes (OVX 30 minutes group, n=7) or 6 hours (OVX 6 hours group, n=8) after MCAO. The ischemic lesion volume was measured using the procedures described in protocol 1.

Statistic analysis

Statistical analysis was performed using Graphpad Prism software (GraphPad Software, Inc, San Diego, CA, U.S.A.). All data are presented as means ± SEM. Ischemic lesion volumes were compared by one-way ANOVA followed by Tukey tests. A probability of <0.05 was considered significant.

Result

Effect of 17β-estradiol on mortality

The lesion protocol used caused inadvertent death of some animals due to an excessively large lesion volume. Deaths prior to the planned 48 hour sampling of animals occurred in 5 OVX and 4 E2-treated rats. These rats fully recovered from surgery and anesthesia and then died within 48 hours of occlusion. These animals were excluded from the study. Interestingly, in this subset of animals, the OVX rats displayed more severe motor deficits and died earlier (<12 h after the occlusion) than the E2-treated rats. The E_2-treated rats were less affected and survived up to 24-36 h after occlusion.

E_2 administration and serum E_2 concentration

A single subcutaneous injection of a pharmacological dose of E_2 (100 μg/kg body weight) rapidly increased plasma E_2 concentrations (Fig.1.). Plasma E_2 concentrations reached 2000 pg/ml by 5 min (the first sampling time,) and remained at this level through 1 hour post-injection then decreased thereafter. At 24 hours after administration, concentrations of E_2 were still elevated (167.9 ± 71.8 pg/ml, Fig. 1.).

The neuroprotective effect of 17β-estradiol are dose dependent when administered 30 minutes after p-MCAO

Permanent occlusion of the MCA resulted in an average total lesion volume of 352 ± 28 mm^3, with the lesion localized primarily in the parietal cortex (223 ± 21 mm^3) and basal ganglia (130 ± 12 mm^3) (Fig. 2 & 4). Subcutaneous injection of 100 μg/kg or 500 μg/kg E_2 at 30 minutes after the permanent occlusion resulted in a total lesion volume of 224 ± 21 mm^3 and 130 ± 31 mm^3, respectively. This corresponds to a reduction in total lesion volume by 36 ± 6% and 63 ± 9%, respectively (Fig. 3.A). This estrogen-mediated protection was observed in both cortex and subcortex regions of the ischemic infarct (Fig.3B & 3C). In the ischemic cortex, sc injection of 100 μg/kg or 500 μg/kg E_2 30 minutes after the occlusion resulted in an average cortical lesion volume of 137 ± 21 mm^3 (a 39 ± 9% reduction) and 66 ± 26 mm^3 (a 71 ± 12% reduction), respectively. In the subcortex, lesion volumes were reduced to 84 ± 6 mm^3 (a 35 ± 5% reduction) at the lower dose of (100 μg/kg) and 73 ± 9mm^3 (a 44 ± 7 % reduction) for the higher dose (500 μg/kg group).

Therapeutic window for E_2 protection

Ischemic lesion volumes were significantly reduced from 352 ± 28 mm^3 in vehicle controls to 212 ± 24 mm^3 (a reduction of 40 ± 7%) when the 1 mg/kg dose of E_2 was administered at 30 minutes after the ischemic insult. At 6 hours after the occlusion, 33% of animals tested (4 of 12 rats) were protected with the 1 mg/kg E_2 dose. In this subgroup, the ischemic lesion volume was reduced from 318 ± 29 mm^3 in vehicle control group to

136.4 ± 28.6mm^3 (57 ± 9% reduction). However, 67%, or 8 out of 12 rats, in the same group were not protected by E2 treatment at 6 hours after the onset of MCAO.

Discussion

The present study demonstrates 3 important characteristics of the potential use of estrogens in treatment of subjects during an ongoing stroke. First, the estrogen neuroprotection is dose-dependent and not associated with toxicity even at high doses. Second, the therapeutic window appears to be at least 6 hours in a subgroup of animals. Finally, subcutaneous administration of an oil formulation produces a rapid and sustained increase in circulating estrogens at concentrations that are neuroprotective for at least 24 hours. Collectively, these data indicate that estrogen formulations and dosing regimens can profoundly protect the brain from ischemic damage.

The oil formulation tested in the present study produced a repaid increase in circulating E_2 concentrations, with high level seen by the first sampling time at 5 min. and levels were maintained above those needed to protect brain tissue [23, 32] for at least 24 hours. In as much as no toxicity was seen with this formulation and mode of dosing E_2, it appears to be a safe therapeutic. Indeed, even in animals that dies as a result of large ischemic lesion produced, the E_2 treatment appears to improve symptoms and survival time.

Our observation of dose-dependent protection with E_2 administered after the onset of MCAO is important since the safety of E_2 have not been established for acute high dose administration. The peak concentrations of E_2 achieved and sustained form about 2 hours with sc administration of 100 μg/ml with this formulation were 2000 pg/ml (about

7 nM). We assume that the 1 mg/ml dose of E_2 would produce 10 times higher peak concentrations (or 70 nM plasma E_2 concentrations). In a variety of in vitro studies these peak concentrations of E_2 are potently neuroprotective and do not exhibit toxicity [1, 2, 11,13,15].

Our observation of a therapeutic window for E_2 administration in an oil formulation of 6 hours for a third of animals tested indicates that estrogens can protect brain tissue through mechanisms that are initiated long after the onset of the ischemic event. A variety of proposed mechanisms for estrogen neuroprotection have been proposed [4, 8, 22], among which are activation of anti-apoptotic proteins [33-36] and preservation of mitochondrial integrity [9, 37]; Our results suggest that estrogen treatment delayed by as much as 6 hours can effect these neuroprotective processes in a way the affords substantial benefit for a subpopulation of subjects.

We observed cortical as well as subcortical protection with delayed estradiol treatment. In pretreatment paradigms, consistent protection of the cortex has been reported [18, 23] while some studies report subcortical protection [21, 38] while others do not [25, 39]. The apparent resistance of the subcortex to E2 protection could be due to at least two factors. First, the basal ganglion does not appear to express estrogen receptors (ER) [40]. This could contribute to the relative resistance of the basal ganglion to E2 treatment through the lack of ER-mediated neuroprotection in this region of the brain, as has been proposed. However, the basal ganglion is an exclusive territory of the middle cerebral artery and as such does not receive blood from collateral arteries, in contrast to the extensive collateral circulation of the cortex [23]. As such, MCAO results in a more severe ischemia in the subcortex than the cortex [39, 41]. Our observation that high doses

of estradiol, even when administered after the onset of the ischemia can protect the subcortex supports the hypothesis that this brain region is estrogen protectable, but subjected to a great insults during MCAO.

In conclusion, we have shown that a subcutaneous oil preparation for E_2 delivery that produces rapid and sustained elevations in circulating E_2 causes a dose-dependent protection from the ischemia for MCAO. This protection is seen for as long as 6 hours after the onset of the ischemia. Collectively, these results suggest that sc estrogen treatment with oil formulations during ongoing strokes may improve neurological outcome.

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Legends for figures

Figure.1. Plasma β-E_2 levels after β-E_2 administration. 7 ovariectomized female Sprague Dawley rats were subcutaneous injected of 100μg/kg E_2 in sesame oil with 2% benzyl alcohol. Blood samples (0.5ml for the first 5 time points and 1 ml thereafter) were taken via cannula at 15-30 minutes prior to administration of the compound (time 0), and 5, 15, 30 minutes and 1, 4, 8, 24 hours after E_2 administration. Plasma was collected and β-E_2 concentration was determined by RIA. Plasma β-E_2 concentration is given in pg/ml: 1nM = 272pg/ml. Mean ± SEM values are depicted.

Figure 2. **A**. Effect of 30 minutes posttreatment with 100 μg/kg & 500 μg/kg of 17β-estradiol or vehicle on ischemic lesion volume induced by MCAO. Treatment was administered by subcutaneous injection 30 minutes after onset of the occlusion. 17β-estradiol significantly decreases total infarct volume by 36.4% ± 6.0% and 63.1% ± 8.9% in 100 μg/kg (n=5) & 500 μg/kg (n=7) group vs. OVX group (n=9), respectively. **B**. Effect of 30 minutes posttreatment with 100 μg/kg & 500 μg/kg of 17 β-estradiol or vehicle on ischemic cortex lesion volume induced by MCAO. Treatment was administered by subcutaneous injection 30 minutes after onset of the occlusion. 17β-estradiol significantly decrease cortex infarct volume by 38.8% ± 9.2% and 70.5% ± 11.8% compared to OVX rats in 100 μg/kg (n=5) & 500 μg/kg (n=7) group vs. control group (n=9), respectively. **C**. Effect of 30 minutes posttreatment with 100 μg/kg & 500 μg/kg of 17 β-estradiol or vehicle on ischemic subcortex lesion volume induced by

MCAO. Treatment was administered by subcutaneous injection 30 minutes after onset of the occlusion. 17β-estradiol significantly decrease subcortex infarct volume by 35.3% ± 4.7% and 43.8% ± 7.2% compared to OVX rats in 100 μg/kg (n=5) & 500 μg/kg (n=7) group vs. control group (n=9), respectively. Depicted are mean ± SEM of the means for the ischemic lesion volume in 7 brain slices. *P<0.05 vs. control. **P<0.01 vs. control. ***P<0.001 vs. control

Figure 3. Effect of 30 minutes or 6 hours posttreatment with 1mg/kg of 17β-estradiol or vehicle on ischemic total lesion volume induced by MCAO. Treatment was administered by subcutaneous injection 30 minutes or 6 hours after onset of the occlusion. At 30 minutes after the occlusion 1 mg/kg of 17β-estradiol (E_2 group n=6) significant decrease the total infarct volume by 40.0% ± 6.9% vs. OVX group (n=7). While after 6 hours 4 of 12 rats in treatment group (E_2 group) still showed a 57.0% ± 9.0% decreased of the total infarct volume vs. OVX group (n=8), and 8 of 12 rats in treatment group did not show protection (E_2 group). Depicted are mean ± SEM of the means for the ischemic lesion volume in 7 brain slices. *P<0.05 vs. control.

Figure 4. Photograph displaying representative brain sections obtained from ovariectomized rats treated 30 minutes after onset of permanent MCA occlusion with 100 μg/kg (middle), 500 μg/kg (right) 17β-estradiol or vehicle (left).

Figure 1.

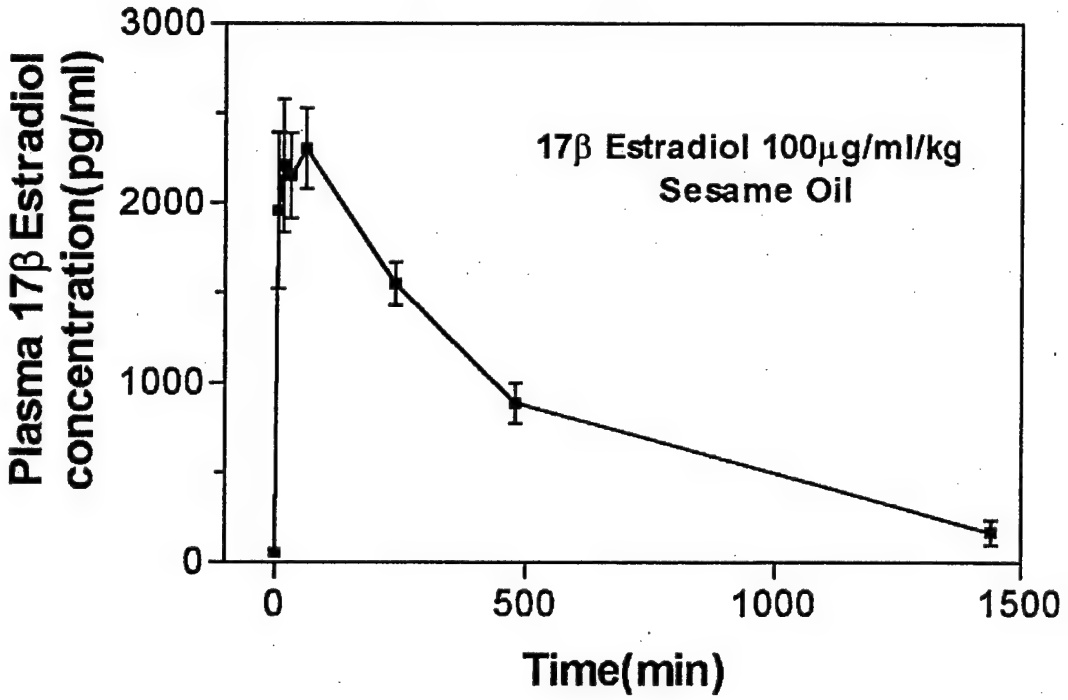
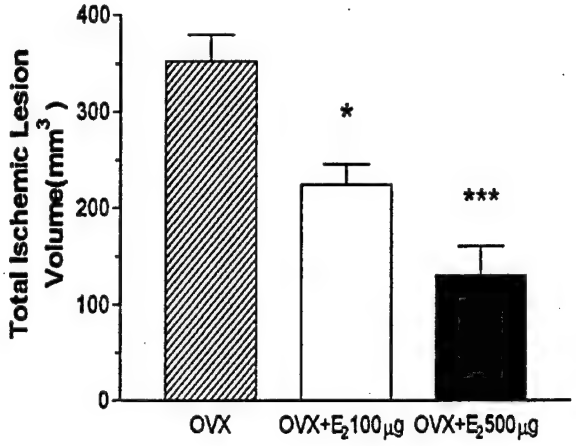
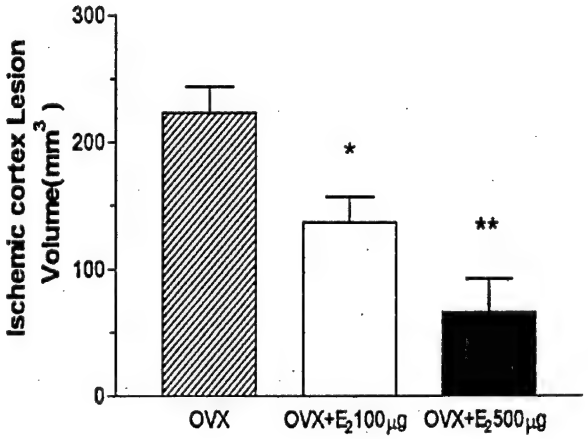

Plasma 17β Estradiol concentration(pg/ml)
3000
2000
1000
0
0
500
1000
1500
Time(min)
17β Estradiol 100μg/ml/kg
Sesame Oil

Figure 2. A


Total Ischemic Lesion Volume(mm3)
400
300
200
100
0
*
***
OVX
OVX+E2100μg
OVX+E2500μg

B


Ischemic cortex Lesion Volume(mm3)
300
200
100
0
*
**
OVX
OVX+E2100μg
OVX+E2500μg

C

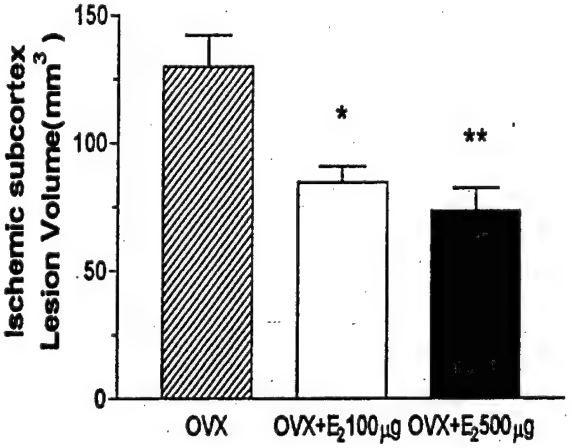

Ischemic subcortex Lesion Volume(mm3)
150
100
50
0
*
**
OVX
OVX+E2100μg
OVX+E2500μg

Figure 3.

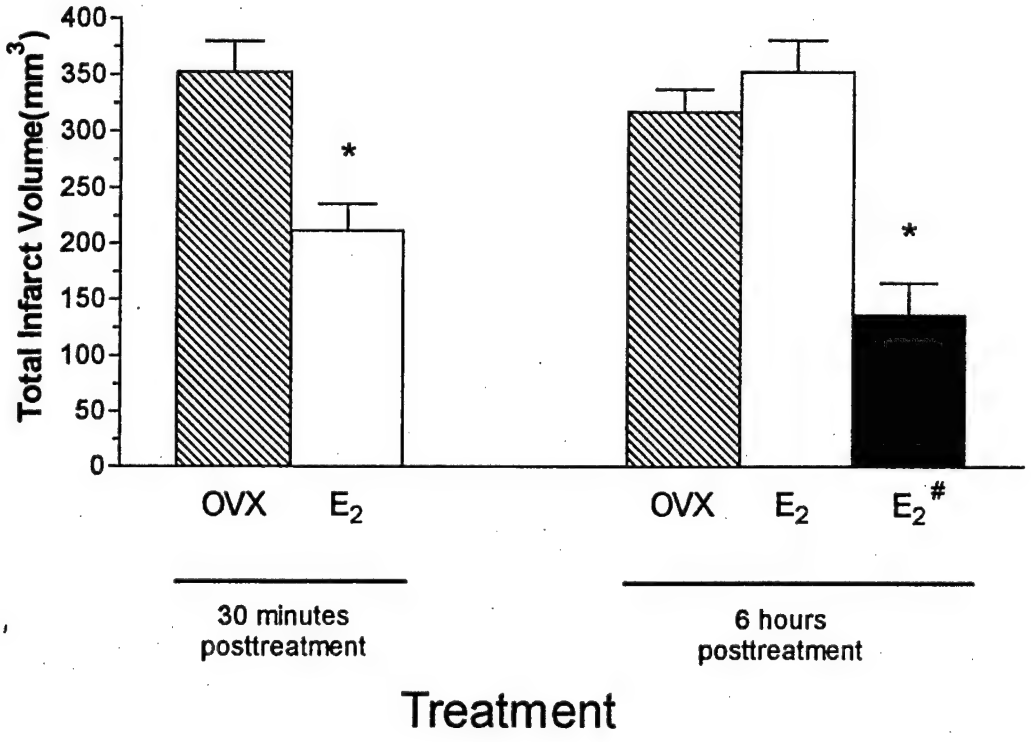
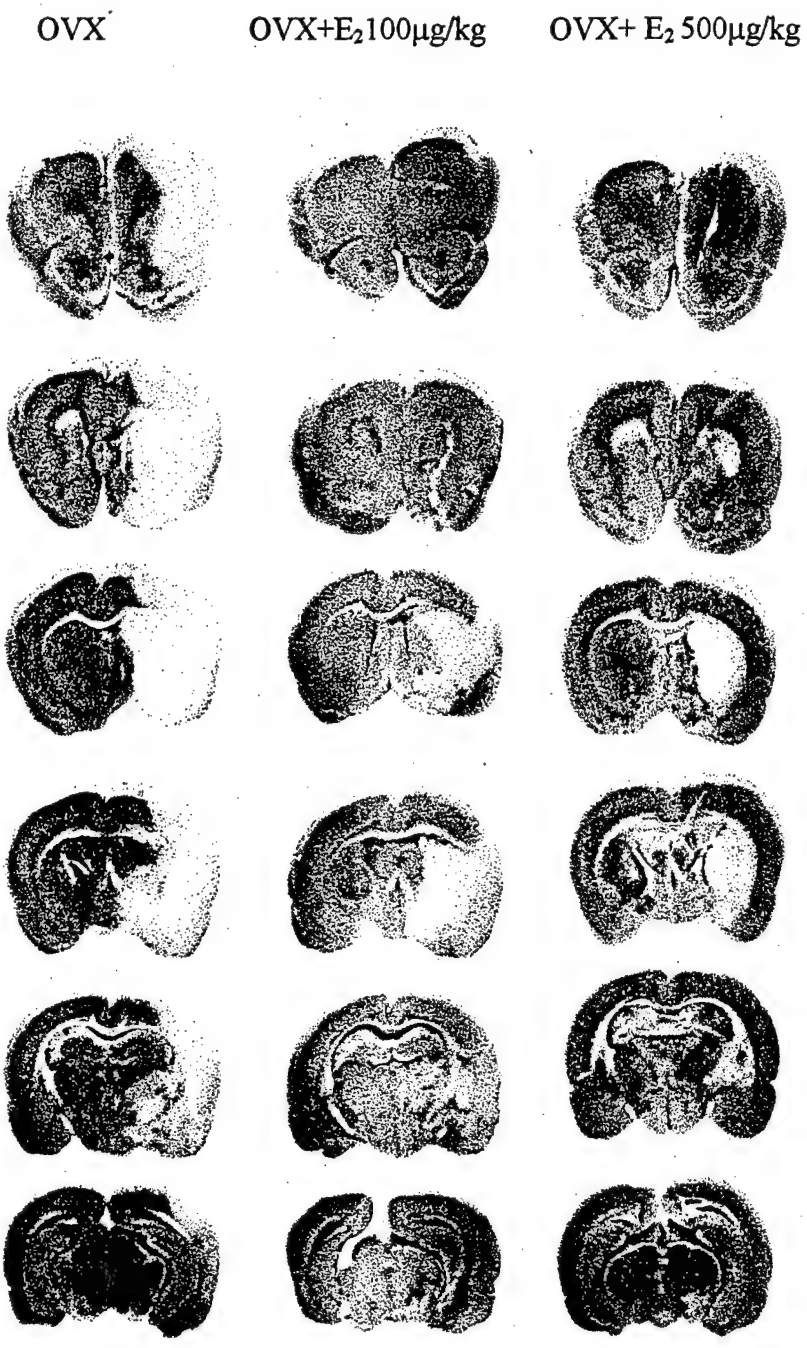

Total Infarct Volume(mm3)
400
350
300
250
200
150
100
50
0
*
*
OVX
E2
OVX
E2
E2#
30 minutes posttreatment
6 hours posttreatment
Treatment

Figure 4. Representative brain section from ovariectomized rats treated 30 minutes after onset of permanent MCAO with vehicle or E_2



Appendix F

The Use of Estrogens and Related Compounds in the Treatment of Damage from Cerebral Ischemia

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Keywords: estrogen, stroke, estrogen receptor, neuroprotection.

Abstract:

There are 750,000 new cases of stroke each year in the U.S. and brain damage from stroke leads to high health care costs and disabilities. Needed, but currently not available, are therapies that can be administered prior to, during or following cerebral ischemia that reduce or eliminate neuronal damage from stroke. To address this issue, we began to assess the neuroprotective effects of estrogens and related compounds in stroke neuroprotection to determine if these compounds had potential for clinical application. First, we demonstrated that 17 β-estradiol (E2) pretreatment exerted potent neuroprotection of the cerebral cortex over a wide dose range and pretreatment interval. Thereafter, we assessed the ability of a variety of non-feminizing estrogens to protect brain tissue from stroke. We observed that pretreatment with 17 α-estradiol; the complete enantiomer of E2 (ENT-E2); 2-adamantylestrone; and the enantiomer of 17-desoxyestradiol, were as effective as E2 in pretreatment protection from stroke damage. These data suggest that non-estrogen receptor mechanisms are involved in brain neuroprotection under our treatment conditions. We then determined if the observed E2 protection could be extended to times after the onset of the cerebral ischemic event. Using a formulation of E2 that rapidly delivers the steroid, a necessary condition for acute therapy of an ongoing stroke, we demonstrated that 100μg E2/kg could protect brain tissue for up to 3 hours after the onset of the stroke. To determine if this therapeutic window could be extended with higher doses of the steroid, we conducted a dose-response assessment of E2 when administered at 6 hours after the onset of the ischemic event. While the effectiveness of the 100 μg E2/kg was reduced at this time interval, higher doses of E2 were effective. E2, at doses of 500 and 1000 μg /kg, reduced infarct volume by over 50% even with this 6 hour delay in treatment. Collectively,

these data indicate that estrogens could prove to be useful therapies in preventing brain damage from strokes.

Stroke ranks as the third leading cause of death and the leading cause of disability in the United States. There are 750,000 new cases of stroke each year in the U.S. and brain damage from stroke leads to high health care costs and disabilities. Stroke patients must not only survive the acute stages of the event, but must then cope with significant physical, mental, and economic stresses associated with neurological damage. Considering the cost both in loss of life and subsequent productivity, the need for effective therapeutic interventions is obvious. The effort to develop effective therapies for stroke achieved several important successes during the past decade. The greatest successes were related to thrombolysis. However, the only federally approved clot-busting medication, tissue plasminogen activator, must be given within three hours of a stroke to be effective. Thrombolysis is severely limited by the need for acute administration. For various reasons, only about 2% of the potentially eligible patients are receiving the treatment [1]. Therapeutic methods are devastately needed, but currently not available, that can be administered prior to, during or following cerebral ischemia that reduce or eliminate neuronal damage from stroke. Neuroprotection, therapeutic interventions that produce enduring benefits by favorably influencing underlying etiology or pathogenesis and thereby forestalling the onset of neuronal damage or decline caused by stroke or other neurodegenerative diseases [2], can be used alone and as an adjunct to therapies designed to improve cerebral circulation such as thrombolytic agents for cerebral arterial thrombosis.

Neuroprotective agents have been developed and tested for nearly all components of the ischemic cascade. Various strategies include free radical scavengers, anti-excitotoxic agents,

apoptosis inhibitors, anti-inflammatory agents, metal ion chelators, ion channel modulators, antisense oligonucleotides, gene therapy and stem cells transplantation. There various agents aim to prevent the progression of ischemic cascade therefore reducing brain damage. Some of these intervene at more than one point in the ischemic cascade. During the last decade, tremendous effort has been made to develop new neuroprotective agents, including estrogens. Neuroprotective effects of estrogens were first suggested by epidemiological studies. Sex differences in the incidence and outcome of stroke suggest that hormonal factors may influence the development and outcome of stroke [3, 4]. Protective effects of estrogen have been widely reported in different types of neuronal cell against different toxicities including serum deprivation, oxidative stress, amyloid β peptide (Aβ) induced toxicity and excitotoxicity [5]. Additionally, the pathological mechanisms that are activated during stoke, include oxidative stress, free radical activity, excitotoxicity, inflammatory response, mitochondrial dysfunction, and apoptosis, are antagonized by estrogens.

Since we first demonstrated that estrogens exert neuroprotective effects in a rodent cerebral ischemia reperfusion model in 1997 [6], there is now abundant *in vivo* evidence for neuroprotection by estrogen. The neuroprotective effects of estrogens have been demonstrated in variety of stroke models by different laboratories, including transient and permanent middle cerebral artery occlusion model (MCAO) [7-9], subarachnoid hemorrhage model [10], global forebrain ischemia model [11-14], photothrombotic focal ischemia model [15], and glutamate induced focal cerebral ischemia model [16]. The neuroprotective effects of estrogens have been demonstrated in females as well as males [17]. The neuroprotective effects of estrogens exert in young aged and middle-aged females [18], as well as reproductively senescent females [19]. Further, these effects of estrogens have been shown despite the presence of diabetes and hypertension [20],

[21]. This indicates that estrogen could be a valuable candidate for the treatment of stroke, in as much as it is effective in both genders and the therpay appears to be resistant to aging, diabetes and hypertension.

Neuroprotective effects of E2 have been demonstrated over a very large range of concentrations. In *in vitro* studies, the effective concentrations for E2-mediated neuroprotection range from low nanomolar (~0.1 nM) to high micromolar (~50 μM) concentrations [5]. High physiological concentrations (low nM) were sufficient to attenuate toxicity in a variety of cell types [5], while significantly higher pharmacological concentrations (low μM) were required to lessen glutamate toxicity. Similarly, large concentration ranges of estrogens, from low physiological concentrations to high pharmacological concentration, has been shown to afford their protective effects in stroke model. Increasing evidence has indicated that neuroprotective effects of estrogens are dose-dependent. No neuroprotection was afford by physiological level of estradiol administered at the time of the onset of ischemia [9], while neuroprotective effects of estrogens were clearly demonstrated by the acute treatment, even post-treatment, with pharmacological doses of estradiol [6, 17, 22, 23]. The different therapeutic time-course for the physiological and pharmacological dose of estrogens also suggest different neuroprotective mechanism afford by different doses.

Many actions of estrogens, including feminizing effects, are mediated by the binding of the steroid to the nuclear ERs, and the binding of the steroid-receptor complex to the ER response element thereby activating transcriptional events. The feminizing effects of estrogens limit their clinical application as a neuroprotectant in men as well as in some women. However, increasing evidence indicated that estrogens could protect brain tissue from ischemic damage via mechanisms independent of estrogen receptors (ER) activation. Several lines of *in vitro* evidence

suggest that the neuroprotective effects of estrogens do not require ER-dependent gene transcription. First, ER antagonists do not attenuate the protective action of E2 in all models of neurotoxicity [24, 25]. Second, neuroprotection of estrogens can occur in the presence of mRNA or protein synthesis inhibitors [26, 27]. Third, non-feminizing estrogens, such as 17α-estradiol [28, 29], complete enantiomer of 17β-estradiol [30], and other phenolic compounds [25] have been shown to be neuroprotective and activate signal transduction cascades associated with neurotrophic effects, such as ERKs at the same doses as 17β-estradiol [31]. The receptor-independent non-genomic action of estrogens was also indicated in the *in vivo* studies. The neuroprotective effects of estrogen have also been demonstrated for several nonfeminizing estrogen analogues, such as 17α-estradiol, the enantiomer of 17β-estradiol and 2-adamantylestrone [6, 30, 32]. These data suggest that non-estrogen receptor mechanisms are involved in brain neuroprotection under these conditions. On the other hand, estrogen receptor-dependent mechanism was also indicated. Dubal et al. [33] reported that ERα knockout, but not ERβ knockout, mice were resistant to the neuroprotective effects of 17 β-E2 administered chronically at low concentrations and concluded that ERα is a necessary mediator of estrogen neuroprotection. However, neuroprotective effects of estrogens have been shown in intact ERα knockout females [34], which have much higher estrogens level than wild type females [35]. Consistently, McCullough et al later demonstrated neuroprotection in ERα knockout mice using pharmacological doses of the estrogens [36]. Together, these data indicated that estrogens could exert neuroprotective effects through both receptor-dependent and –independent mechanism depending upon the dose of estrogen administered. The nonfeminizing estrogen analogues with neuroprotective activity but lacking estrogenic activity in peripheral estrogen responsive tissues could be applied in both males and females for whom estrogen therapy is contraindicated.

At pharmacological doses, neuroprotective effects of estrogens have been demonstrated upon acute pre-treatment, as well as treatments initiated after the onset of the ischemic insult [6, 17, 23]. We demonstrated that 17β-estradiol exerts neuroprotective effects when administered after an ischemic insult, with a therapeutic window of about 3 hours at the dose of 100μg/kg (Figure 1) [22]. However, the dose-dependency of estrogen protection after the onset of a stroke is not known. We conducted a dose-response assessment of 17β-estradiol when administered at 6 hours after the onset of the ischemic event to determine if our previously reported 3 hours therapeutic window [22] could be extended with higher doses of the steroid. Encouragingly, 17β-estradiol treatment at 500μg/kg and 1000μg/kg still exert neuroprotective effects against the cerebral ischemic damage when administered 6 hours after permanent middle cerebral artery occlusion (Figure 2). Given the effectiveness of thrombolysis and the neuroprotective effectiveness of estrogen for both the ischemic and reperfusion phases, combination of reperfusion enhancing agents with estrogens could greatly improve the outcome of stroke. The potential approach to prolonging the therapeutic time window for successful thrombolysis would be to give estrogen as a neuroprotectant before, during, or after thrombolytic therapy. It is likely that this combination therapy will restore blood flow, halt or reverse the cascade of neuronal damage, and will be used to achieve effective stroke care in the future.

Although the application of estrogens for treatment of stroke required further clinical studies, a plethora of data support a direct neuroprotective role for estrogens. Given the proven clinical safety of this steroid, estrogen therapy may be useful in treating acute cerebral ischemia. Further, the efficacy of non-feminizing estrogen analogues suggest that these compounds may be clinically useful for prevention of cerebral damage in men or women for whom estrogen therapy is contraindicated. However, one cannot assume that estrogen administration will always

improve outcome in cerebral ischemia. The neuroprotective effects of estrogens are both dose-dependent and damage severity-dependent. The mechanisms that contribute to the loss of neuroprotection at the high dose of 17β-estradiol (Figure 2) are not clear. It is likely that some side effects were induced by these very high doses of 17β-estradiol, which abolish or mask its neuroprotective actions.

Acknowledgements

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Figure 1. Effects of 17β-estradiol treatment on lesion volume following permanent MCAO in ovariectomized rats. Female Charles Rivers Sprague-Dawley rats (225-250g, Wilmington, MA) were maintained in laboratory acclimatization for three days prior to ovariectomy. Bilateral ovariectomy was performed under methoxyflurane inhalant anesthesia. The animals were subjected to permanent MCAO two weeks after ovariectomy. 17β-estradiol was administered by simultaneous intravenous injection (100μg E2/kg) and subcutaneous implantation of an E2 pellet at ½ (n=8), 1 (n=6), 2 (n=7), 3 (n=6) or 4 (n=9) hours after MCAO. The animals were decapitated two days after MCAO. The brain was sliced and stained with 2,3,5-triphenyltetrazolium chloride for lesion volume analysis. INT = ovary intact rats, OVX = ovariectomized rats treated with saline and empty pellet implantation. Depicted are the means ± SEM. *$p<0.05$ vs. OVX & INT.

Figure 2. Dose-dependent neuroprotection of 17β-estradiol when administered at 6 hours after permanent MCAO. Female Charles Rivers Sprague-Dawley rats (250g, Wilmington, MA) were maintained in laboratory acclimatization for three days prior to ovariectomy. Bilateral ovariectomy was performed under halothane anesthesia. The animals were subjected to permanent MCAO two weeks after ovariectomy. 17β-estradiol formulated in sesame oil was administered by subcutaneous injection at 6 hours after MCAO at the dose of 100 (n=9), 500 (n=10), 1000 (n=10), 2000 (n=9) or 5000 μg/kg (n=9), respectively. OVX animals received sesame oil as control (n=10). The animals were decapitated 24 hours after MCAO. The brain was sliced and stained with 2,3,5-triphenyltetrazolium chloride for lesion volume analysis. Depicted are the means ± SEM. *$p<0.05$ vs. OVX.

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Figure 1.

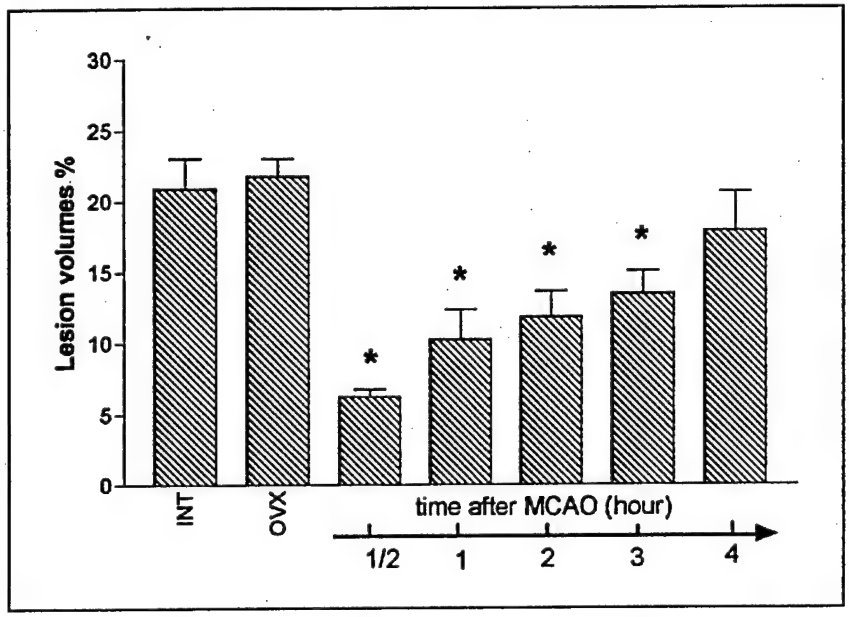
Lesion volumes %
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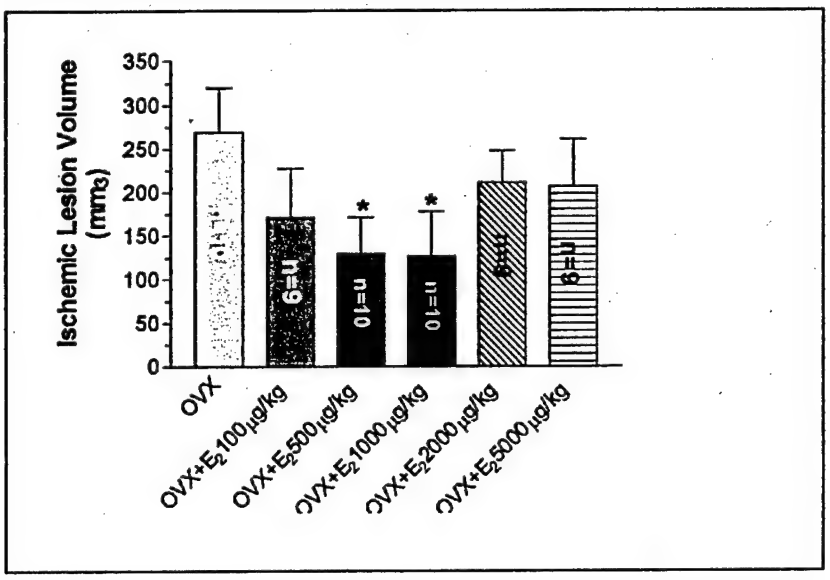
Figure 2.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Appendix G

17-β Estradiol Can Reduce Secondary Ischemic Damage and Mortality of Subarachnoid Hemorrhage

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Summary: Subarachnoid hemorrhage (SAH) is a unique disorder commonly occurring when an aneurysm ruptures, leading to bleeding and clot formation, with a higher incidence in females. To evaluate the influence of 17-β estradiol (E2) in the outcome of subarachnoid hemorrhage, SAH was induced by endovascular puncture of the intracranial segment of internal carotid artery in 15 intact females (INT), 19 ovariectomized females (OVX), and 13 ovariectomized female rats with E2 replacement (OVX + E2). Cerebral blood flow was recorded before and after SAH. All animals were decapitated immediately after death or 24 hours after SAH for clot area analysis. Brains were sliced and stained with 2,3,5-triphenyltetrazolium chloride (TTC) for secondary ischemic lesion analysis. The cortical cerebral blood flow (CBF), which was measured by a laser–Doppler flowmeter, decreased to 29.6% ± 17.7%, 22.8% ± 8.3%, and 43.5% ± 22.9% on the ipsilateral side ($P = 0.01$), and decreased to 63.4% ± 14.1%, 57.4% ± 11.0%, and 66.6% ± 17.9% on the contralateral side ($P = 0.26$) in INT, OVX, and OVX + E2, respectively. The subcortical CBF, which were measured by the H_2 clearance method, were 7.77 ± 12.03, 7.80 ± 8.65, and 20.58 ± 8.96 mL 100 g^{-1} min^{-1} on the ipsilateral side ($P < 0.01$), and 21.53 ± 2.94, 25.13 ± 3.01, and 25.30 ± 3.23 mL 100 g^{-1} min^{-1} on the contralateral side in INT, OVX, and OVX + E2, respectively. The mortality was 53.3%, 68.4%, and 15.4% in INT, OVX, and OVX + E2, respectively ($P = 0.01$), whereas no significant difference in clot area was noted among the groups. The secondary ischemic lesion volume was 9.3% ± 8.4%, 24.3% ± 16.3%, and 7.0% ± 6.4% in INT, OVX, and OVX + E2, respectively ($P < 0.01$). This study demonstrated that E2 can reduce the mortality and secondary ischemic damage in a SAH model without affecting the clot volume. **Key Words:** Estrogens—Subarachnoid hemorrhage—Ischemia—Neuroprotection.

Stroke is the third most common cause of death in the adult population in the United States, after ischemic heart disease and all forms of cancer (Camarata et al., 1994). Subarachnoid hemorrhage (SAH) accounts for approximately 10% of all strokes (Selman et al., 1999). However, SAH affects a younger population and results in death in more than 50% of subjects, most of whom die within the first 24 hours. Subarachnoid hemorrhage accounts for more premature mortality than ischemic stroke (Broderick et al., 1994; Zhang et al., 1998). Subarachnoid hemorrhage can result in vascular changes such as acute vasospasm and intracranial hypertension, which lead to decrease of cerebral perfusion pressure and cerebral blood flow (CBF). All of these can contribute to secondary ischemic damage after SAH. Histologic studies of brains of patients who died shortly after SAH show extensive ischemic damage, and secondary ischemia has been reported to be one of the major causes of death shortly after SAH (Adams et al., 1981).

Unlike other kinds of strokes, aneurysmal SAH occurs more frequently in women than in men (Davis, 1994). Gender differences in the outcome of SAH are controversial, and the influence of the female sex hormone is unclear (Kongable et al., 1996; Simpson et al., 1991; Johnston et al., 1998). However, estrogens have been found to exert neuroprotective effects in models of ischemic stroke both *in vitro* and *in vivo* (Simpkins et al., 1997; Alkayed et al., 1998; Dubal et al., 1998; Zaulyanov et al., 1999; Yang et al., 2000). Whether estrogens exert similar protective effects in SAH as in ischemia is currently unknown. The purpose of this study was to

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determine whether 17β-estradiol (E2) influences the outcome of SAH and, if so, whether the influence relates to the ischemia associated with SAH.

MATERIALS AND METHODS

Preparation of animals

Female Charles River Sprague–Dawley rats (250 g, Wilmington, MA, U.S.A.) were maintained in laboratory acclimatization for 3 days before ovariectomy. Bilateral ovariectomy was performed two weeks before SAH under methoxyflurane inhalant anesthesia. All animal procedures were approved by the University of Florida Animal Care and Use Committee.

E2 administration and serum concentration

To obtain a sustained stable elevation in serum E2 concentration, implantation of a Silastic® pellet containing the steroid was used. To assess serum concentrations of E2 after this treatment regimen, a group of OVX animals ($n = 5$) was anesthetized with methoxyflurane inhalant and a control blood sample was taken through the jugular vein. Then a 30-mm-long Silastic® tube (1.57 mm inner diameter, 3.18 mm outer diameter) containing E2 (4 mg/mL in corn oil) was implanted subcutaneously in 5 OVX animals. Animals were returned to their cages and blood samples then were taken through the jugular vein at 24 and 48 hours after steroid administration, under methoxyflurane inhalant anesthesia. Serum was separated from blood cells by centrifugation and stored frozen (−20°C). Serum E2 concentrations were determined using duplicate serum aliquots in an ultrasensitive estradiol radioimmunoassay kit (Diagnostic Systems Lab, Webster, TX, U.S.A.).

Endovascular subarachnoid hemorrhage model

Animals were anesthetized by intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). Rectal temperature was monitored and maintained between 36.5°C and 37.5°C during the procedure. With the aid of an operating microscope, the left common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed through a midline cervical skin incision. A 3–0 monofilament suture with a blunt tip was introduced into the ICA through the ECA lumen, and advanced until resistance was encountered. The distance between the CCA bifurcation and the resistive point was approximately 1.8 cm. The suture was advanced another 5 mm and then withdrawn immediately. The CCA and ICA were coagulated and the skin incision was closed.

Measurement of regional cerebral blood flow

A laser–Doppler flowmeter (LDF) was used for cortical CBF measurements. The scalp was incised on the midline, and bilateral 2-mm burr holes were drilled 1.5 mm posterior and 4.0 mm lateral to the bregma. The dura was left intact to prevent cerebral spinal fluid leakage. Laser–Doppler flowmeter probes held in place by a micromanipulator were stereotaxically advanced to gently touch the intact dura mater. The lower stable readings were obtained and recorded for at least 10 minutes from both sides (baselines measurement) (Dubal et al., 1998; Cholet et al., 1997). For each animal, the lower CBF reading was recorded at the same sites within 30 minutes after SAH. The CBF values were calculated and expressed as a percentage of the baseline values. Cerebral blood flow values reported represent the mean ± SD for the average of the CBF recordings obtained.

Hydrogen clearance method was used for subcortical CBF measurement: two Teflon-coated platinum electrodes held in place by a micromanipulator were stereotaxically advanced to 3 mm posterior, 0.5 mm lateral to the bregma, and 4.0 mm deep into the subcortical region on both sides (He et al., 1995). For each animal, CBF were recorded 30 minutes after SAH.

Measurement of clot and lesion volume

Each group of animals was decapitated immediately after death or 24 hours after SAH, the brain was removed, and the base of the brain was photographed by a digital camera (Sony MVC-FD5, Tokyo, Japan) for measurement of the clot area. Then the brain was placed in a metallic brain matrix (Harvard, Holliston, MA, U.S.A.) for tissue slicing. Two-millimeter sections were made beginning at 3, 5, 7, 9, 11, and 13 mm posterior to the olfactory bulb. Each slice was incubated for 30 minutes in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) in physiologic saline at 37°C and then fixed in 10% formalin. The stained slices were photographed and subsequently measured for the surface area of the slices and the ischemic lesion (Image-Pro Plus 3.0.1, Silver Springs, MD, U.S.A.). Clot area was calculated as the percentage of the base of the brain covered by clot to represent the clot size. Ischemic lesion volume was calculated as the sum of the areas of the ischemic lesion across the six slices divided by the total cross-sectional area of these six brain slices.

Experimental protocol

Forty-seven animals were placed into 3 groups: 15 intact females (INT), 19 ovariectomized females (OVX), and 13 ovariectomized females with estrogen replacement (OVX + E2), respectively. In OVX + E2 animals, a 30-mm-long Silastic® tube containing E2 (4 mg/mL oil) was implanted subcutaneously 24 hours before SAH under methoxyflurane inhalant anesthesia. Intact and OVX animals received a Silastic® tube containing oil as a control.

Twenty-four hours later, after a baseline CBF reading was obtained, SAH was induced in each animal. Cerebral blood flow was recorded bilaterally at the same sites within 30 minutes after SAH. Then the animals were returned to their home cages under careful observation. Each animal was decapitated for clot and ischemic volume analysis immediately after death or 24 hours after SAH.

Subcortical CBF was measured in 15 animals 30 minutes after SAH—5 each for INT, OVX, and OVX + E2—using the hydrogen clearance method. The right femoral artery was catheterized for blood pressure monitor in each animal. Mean arterial pressure was recorded before, immediately after, and 30 minutes after SAH.

Statistic study

Statistical analyses were performed using SAS Software (SAS Institute, Cary, NC, U.S.A.). Paired *t*-tests were performed to assay serum concentration of E2 treatment after implantation. The authors compared the clot areas, lesion volumes, and CBF from the three groups. For each comparison, *P* values from one-way analysis of variance were provided. Chi-squared test was used to compare the mortality rate among the three groups.

RESULTS

E2 administration and serum E2 concentration

Implantation of a 30-mm E2 (4 mg/mL corn oil) pellet maintained stable serum E2 concentrations for at least 48 hours after administration. E2 concentration was 24.9 ± 6.6 pg/mL and 31.3 ± 11.5 pg/mL at 24 and 48 hours after administration, respectively, compared with 3.5 ± 1.2 pg/mL in OVX animals ($P < 0.01$) (Fig. 1).

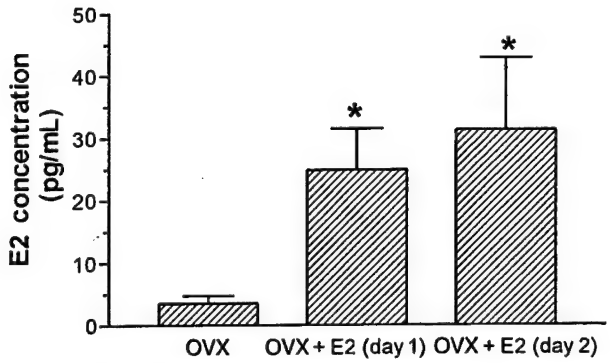

FIG. 1. Serum E2 concentrations after treatment. E2 administration: subcutaneous implantation of a 30-mm-long Silastic® pellet filled with E2 oil (4 mg/mL). *OVX versus OVX + E2 (day 1), $P = 0.0002$; OVX versus OVX + E2 (day 2), $P + 0.0028$. Graph shows mean ± SD for 5 animals per group.

Effect of E2 on subarachnoid hemorrhage mortality

Most of the animals began to recover from the anesthetic at approximately 3 hours after SAH. Mean arterial blood pressure was 90.25 ± 9.00, 93.40 ± 11.41, and 97.17 ± 6.77 mm Hg in INT, OVX, and OVX + E2 before SAH, respectively. Blood pressure increased to 123.20 ± 6.61, 121.40 ± 9.34, and 114.70 ± 16.12 mm Hg immediately after SAH, then returned to 94.00 ± 17.72, 91.50 ± 17.23, and 81.80 ± 5.50 mm Hg 30 minutes after SAH in INT, OVX, and OVX + E2, respectively. No significant differences were noted among groups. The mortality was 53.3%, 68.4%, and 15.4% in INT, OVX, and OVX + E2 groups, respectively ($P = 0.01$). In the INT group, 3 animals died within 6 hours after SAH and 5 died between 6 and 24 hours after SAH. In the OVX group, 4 animals died within 6 hours and 9 died between 6 and 24 hours after SAH. In the OVX + E2 group, 1 each died within 6 hours and between 6 and 24 hours after SAH, respectively (Table 1).

Effect of E2 on clot area and secondary ischemic lesion volume

Clotted and unclotted blood was found around the circle of Willis distributed to both sides of the brain with the majority on the ipsilateral side. Blood was also found as a thin layer overlying both sides of the cortex. No clotted blood was found intracerebrally (Fig. 2). No significant differences of clot area were noted among INT, OVX, and OVX + E2 groups, which were 6.6% ± 5.0%, 5.9% ± 4.5%, and 7.6% ± 3.2%, respectively, of the brain base surface ($P = 0.56$) (Fig. 3).

TABLE 1. *Effects of ovarian steroids environment on mortality and time of death in rats subjected to SAH*

Group	Mortality	Died within 6 hours	Died between 6 and 24 hours
INT (n = 15)	8 (53.3%)	3 (20.0%)	5 (33.3%)
OVX (n = 19)	13 (68.4%)	4 (21.0%)	9 (47.4%)
OVX + E2 (n = 13)	2 (15.4%)	1 (7.7%)	1 (7.7%)

INT, intact female; OVX, ovariectomized female; OVX + E2, ovariectomized female with E2 replacement. OVX versus OVX + E2, $P = 0.01$; INT versus OVX + E2, $P = 0.09$; INT versus OVX, $p = 0.59$.

The secondary ischemic lesion was confined to the ipsilateral somatosensory cortex and basal ganglia in most of the animals. Contralateral somatosensory cortex was also involved in 8 of the 19 OVX (42.1%), 2 of the 15 INT (13.3%), and 3 of the 13 OVX + E2 animals (23.1%). The secondary ischemic lesion volume was 9.3% ± 8.4%, 24.3% ± 16.3%, and 7.0% ± 6.4% in INT, OVX, and OVX + E2 groups, respectively ($P < 0.01$) (Fig. 4).

Effect of E2 on cerebral blood flow after subarachnoid hemorrhage

The ipsilateral cortical CBF was reduced to 29.6% ± 17.7%, 22.8% ± 8.3%, and 43.5% ± 22.9% of the baseline in INT, OVX, and OVX + E2, respectively ($P = 0.01$) (Fig. 5). The contralateral cortex CBF was reduced to 63.4% ± 14.1%, 57.4% ± 11.0%, and 66.6% ± 17.9% of baseline in INT, OVX, and OVX + E2, respectively ($P = 0.26$) (Fig. 5).

The subcortical CBF, which were measured by H_2 clearance, were 7.77 ± 12.03, 7.80 ± 8.65, and 20.58 ± 8.96 mL 100 g^{-1} min^{-1} on the ipsilateral side ($P < 0.01$), and 21.53 ± 2.94, 25.13 ± 3.23, and 25.30 ± 3.01 mL 100 g^{-1} min^{-1} on the contralateral side, in INT, OVX, and OVX + E2, respectively (Fig. 6).

DISCUSSION

Subarachnoid hemorrhage is a unique disorder and a major clinical problem that commonly occurs when an aneurysm in a cerebral artery ruptures leading to bleeding, clot formation, early morbidity, and mortality. The incidence of SAH in females is greater than in males. But the influence of the female sex steroid on SAH outcome remains controversial. This study evaluated the influence of the female sex steroid on SAH outcome, and for the first time demonstrated that 17β-estradiol (E2), which is the most active natural estrogen, can reduce the mortality and secondary ischemic damage in a SAH model.

Several studies have demonstrated that E2 exerts neuroprotective effects in ischemic stroke (Simpkins et al., 1997; Shi et al, 1997; Dubal et al., 1998; Toung et al., 1998; Chen et al., 1998; Hawk et al., 1998; Wang et al., 1999; Rusa et al., 1999; Yang et al., 2000). But the effect of E2 on secondary ischemic damage of SAH, which has been demonstrated both in clinical and experimental studies and is one of the major reasons of death shortly after SAH, is unknown. The secondary ischemic damage can be caused by severe CBF reduction immediately after SAH, or processes involving vasospasm and edema leading to the reduction of CBF, or both (Warnell, 1996).

The endovascular SAH model, which uses focal puncture of the internal carotid artery, results in extensive

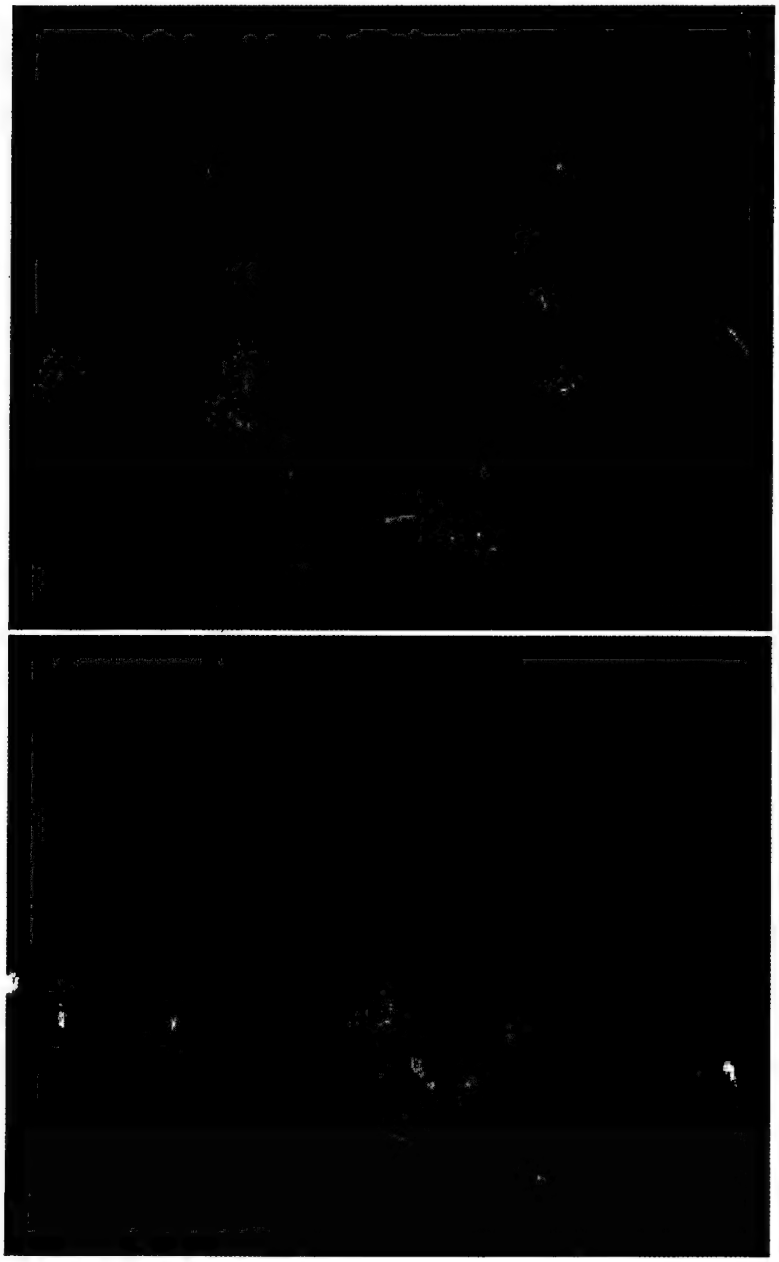

FIG. 2. Figure depicts sham **(top)** and blood distribution in subarachnoid hemorrhage **(bottom)**. Clotted and unclotted blood distributes to both sides of the brain with the majority on the ipsilateral side. Blood was also found as a thin layer overlaying both sides of the cortex.

distribution of blood throughout the subarachnoid space. This blood distribution is similar to that observed with SAH in human subjects and makes the studies of acute pathophysiologic changes of SAH, such as secondary ischemic damage, more clinically relevant (Veelken et al., 1995; Bederson et al., 1995; Matz et al., 1996). Diffusion magnetic resonance imaging study in this SAH model has shown that the acute secondary ischemic damage is confined primarily to the ipsilateral somatosensory cortex and basal ganglia and is involved in the contralateral somatosensory cortex in some of the cases, which was consistent with the authors' secondary ischemia result (Busch et al., 1998). Asymmetric ischemic damage could have resulted from the asymmetric clot distribution in this model, because there is a direct relation between the location of the thickest blood clots and the most severe vasospasm (Camarata et al., 1994).

Deprivation of ovarian steroids, which result from ovariectomy, increased secondary ischemic damage in the current study. In contrast, replacement of E2 in OVX animals decreased the secondary ischemic damage to the level below that of normal females. This study demonstrated that E2 exerted the similar neuroprotective effects in secondary ischemic damage of SAH as previously reported for ischemic stroke (Simpkins et al., 1997; Shi et al., 1997; Dubal et al., 1998; Toung et al., 1998; Chen et al., 1998; Hawk et al., 1998; Wang et al., 1999; Rusa et al., 1999; Yang et al., 2000). The neuroprotective ef-

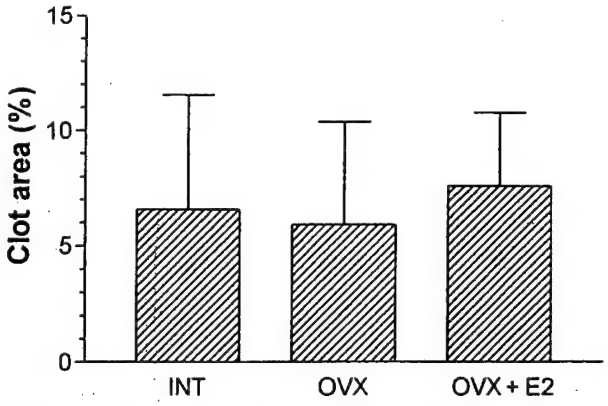

FIG. 3. Clot volumes in INT (n = 15), OVX (n = 19), and OVX + E2 (n = 13). No significant differences were noted among the groups ($P = 0.5617$). Graph shows mean ± SD.

fects of endogenous ovarian steroids or exogenous estradiol on secondary ischemic damage are of the same magnitude as is reported for primary ischemia. Thus, it appears that both endogenous and exogenous E2 exert neuroprotective effects in secondary ischemic damage of SAH in a manner that is not associated with changes of clot in SAH.

The neuroprotective mechanisms of E2 are not yet elucidated, although both direct neuroprotective action on neurons and indirect effects on the cerebral vasculature are possible. Direct effects can include reduction in reactive oxygen species that accumulate during ischemia (Hall et al., 1991), blockade of excitatory amino acid toxicity (Singer et al., 1996; Weaver et al., 1997), modulation of calcium homeostasis (Collins et al., 1993, 1996; Mermelstein et al., 1996; Chen et al., 1998), induction of antiapoptotic protein (Singer et al., 1998; Pike, 1999), and/or enhancement of brain glucose uptake (Shi et al., 1997; Alvarez et al., 1997). Estrogens classically exert their effects by a nuclear estrogen receptor mechanism of action. It is still controversial whether the neuroprotective effect of estrogens is receptor-dependent or -independent. Several lines of evidence suggest that these neuroprotective effects are not a result of the classical estrogen receptor-mediated mechanism. First, tamoxifen, a mixed estrogen receptor agonist/antagonist, does not block the neuroprotective effect of 17-β E2 in serum-deprived SK-N-SH neuroblastoma cells (Green et al., 1997; Culmsee et al., 1999). Second, 17-α E2 has been demonstrated to have neuroprotective efficacy and potency similar to 17-β E2, although 17-α E2 binds only weakly to the estrogen receptors (Green et al., 1997). However, several studies demonstrated that estrogen's neuroprotective effects are receptor-dependent. ICI 182780, an estrogen receptor antagonist, can prevent E2 from protecting against cell death (Wilson et al., 2000). It seems that estrogens may act by multiple mechanisms. E2 could protect from the secondary ischemic damage through similar mechanisms as in ischemic stroke; however, other mechanisms could be operative. Estrogen has been reported to exert both neuroprotective and flow-preserving effects (Toung et al., 1998), but the ischemic protective effects of estrogens seem independent of flow-preserving effects (Dubal et al., 1998; Wang et al., 1999). The current results suggest that estrogen's protective effects on ischemia are flow-independent, because the secondary ischemia lesion volumes were also significantly reduced in the INT group even though both the cortical and subcortical residue CBF were at same level as that in OVX group.

In the E2 replaced animals, both cortical and subcortical residual CBF on the ipsilateral side of the SAH remained greater than that of the OVX and INT group after SAH, whereas no significant difference was noted on the contralateral side among groups. The reduction of CBF is closely related to the presence of blood and blood breakdown products within the perivascular spaces, acting either directly upon the cerebral vessel wall or perhaps more indirectly through perivascular nerves and central brain stem afferent connections to produce an acute vasospasm (Jackowski et al., 1990). Using the similar SAH model, Bederson et al. (1998) have shown that the internal circumference of internal carotid artery and anterior cerebral artery decreased to approximately 50% at 60 minutes after SAH. So the flow-preserving effect of estrogen could partly result from its interaction on vasospasm. Nitric oxide (NO) has been demonstrated to be related to the vasospasm after SAH (Sobey and Faraci, 1998; Sayama et al., 1998), and estrogen appears to alter mygenic tone by increasing cerebrovascular NO production, or action, or both (Geary et al., 1998). Estrogen can also cause the rapid dilation of blood vessels

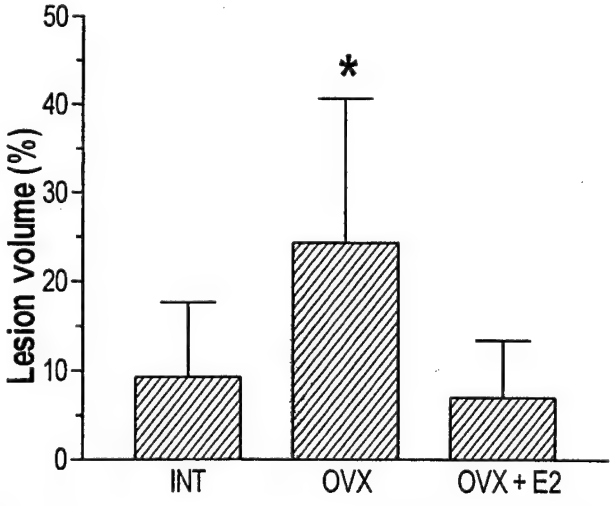

FIG. 4. Secondary ischemic lesion volumes in INT (n = 15), OVX (n = 19), and OVX + E2 (n = 13). Lesion volume of OVX was significantly greater than that of INT and OVX + E2 ($P = 0.0003$). Graph shows mean ± SD.

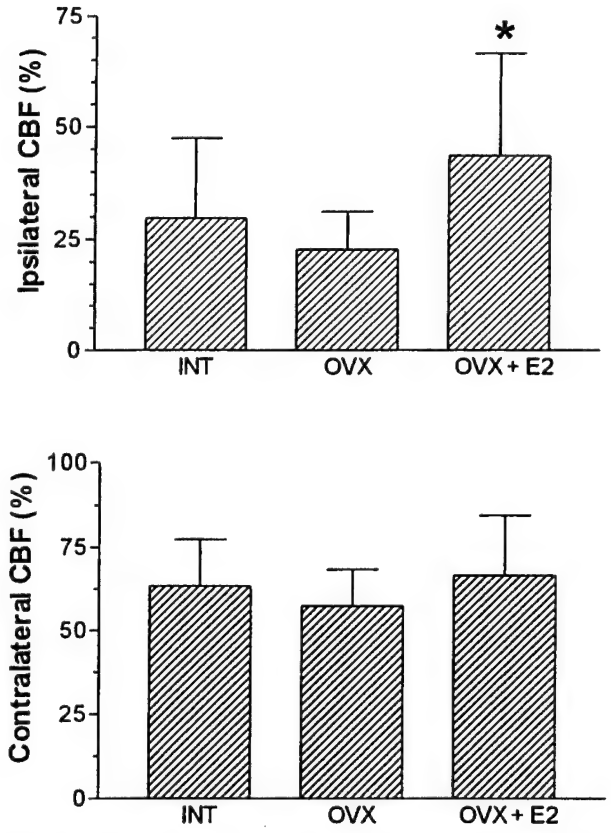

FIG. 5. Cortical cerebral blood flow (CBF) in both sides of INT, OVX, and OVX + E2. In the left side (ipsilateral), cortical CBF of OVX + E2 was significantly greater than that of OVX and INT (P = 0.0119). In the right side (contralateral), no significant differences were noted among the groups. Graph shows mean ± SD.

by stimulating endothelial nitric oxide synthase (Shaul, 1999). The current results suggest that the blood flow-preserving effect could only be induced by exogenous estrogen replacement. Because the exogenous estrogen level in the current study was 24 to 31 pg/mL, which is within the physiologic range (Butcher et al., 1974; Nequin et al., 1979), the lack of blood flow preservation in INT rats was not because of circulating estradiol levels. One explanation for the lack of this effect in endogenous steroids is the presence of progestin in intact female, which varied from 5 to 82 ng/mL, depending on the stage of the estrous cycle, in INT females and less than 5 ng/mL in OVX females (Butcher et al., 1974; Nequin et al., 1979; Murphy et al., 2000). Sarrel (1999) has shown that the vasodilation response to estrogen in women is blunted by progestin. However, the observation that secondary ischemia is similarly reduced in both INT and OVX + E2 rats suggests that progestin is not influencing the neuroprotective effects of estrogen and that the observed blood flow-preserving effects of exogenous estrogens are not the primary neuroprotective mechanism. In other words, in the SAH model, estrogen's neuroprotective effects are flow-independent.

Although both endogenous female steroids and exogenous E2 reduce secondary ischemic damage in SAH, endogenous female steroids (INT group) were not associated with a significant reduction of mortality, whereas E2 replacement markedly reduced mortality. The observation of effects of endogenous ovarian steroids on mortality is consistent with the clinical studies of the outcome of SAH. Kongable et al. (1996) showed that the SAH outcome of women and men is the same even though women were older and harbored more aneurysms. Simpson et al. (1991) found that men have a high risk of unfavorable outcome after SAH. In contrast to the above studies, Johnston et al. (1998) found the mortality of SAH was 62% greater in females than in males. Overall, sex differences in the clinical outcome of SAH are unresolved. The different effects of exogenous estrogen and endogenous female steroids on CBF could attribute to the different mortality between the INT and the OVX + E2 group. The vasodilation effect of estrogens can be reduced by progestin (Sarrel, 1999), suggesting that a progestin blockade of this important action of estrogens could account, in part, for the increased mortality of

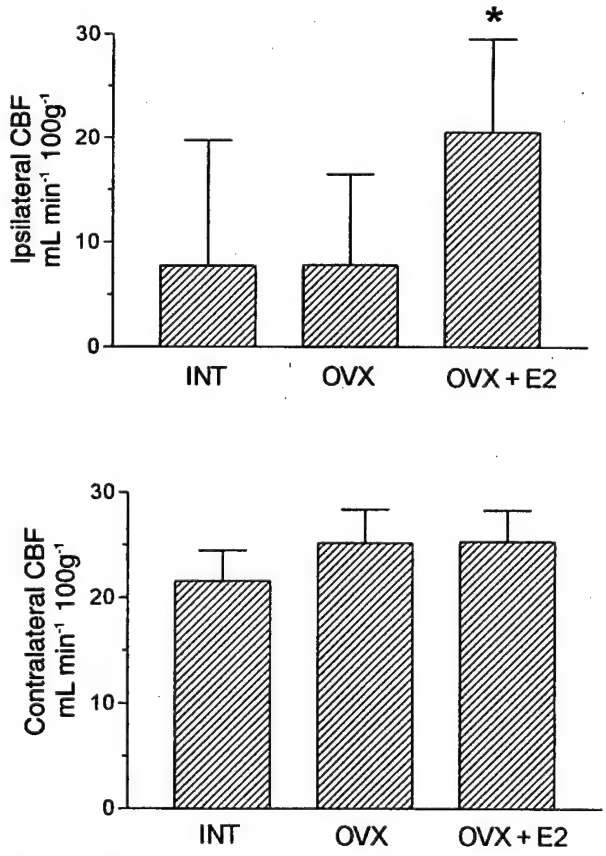

FIG. 6. Subcortical cerebral blood flow (CBF) in both sides of INT, OVX, and OVX + E2. In the left side (ipsilateral), subcortical CBF of OVX + E2 was significant greater than that of OVX and INT (P < 0.01). In the right side (contralateral), no significant differences were noted among the groups. Graph shows mean ± SD.

intact females. In addition, hydrocephalus, which occurs in 20% of patients with SAH and is associated with additional morbidity and mortality, is related to vasospasm after SAH (Black, 1986; Suarez-Rivera, 1998). Further studies of ovarian steroids and their interaction in acute vasospasm and vasospasm-related hydrocephalus are needed to explain the difference of endogenous female steroids and exogenous E2 replacement in mortality after SAH.

In summary, the current study demonstrated that E2 could reduce the secondary ischemic damage and mortality of SAH by exerting neuroprotective effects. These effects are not associated with the change of the clot volume in SAH.

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Appendix H

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Proestrus levels of estradiol during transient global cerebral ischemia improves the histological outcome of the hippocampal CA1 region: perfusion-dependent and-independent mechanisms

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Abstract

We conducted this study to determine whether high physiological levels of estradiol (proestrus) could protect the hippocampal CA1 neurons following transient global ischemia. Ovariectomized or ovary-intact female rats were subjected to 20 min of ischemia and allowed to survive for 96 h. Estradiol was administered subcutaneously in a group of ovariectomized rats 24 h before ischemia induction. Ending serum estrogen levels were correlated to cerebral blood flow (CBF), histologic assessment and immunofluorescent caspase-3 active peptide (C-3AP) positive cell count. Estradiol administration significantly improved CBF in the hippocampus (compared with intact or ovariectomized rats) but not in the parietal cortex. No significant differences in CBF between intact or ovariectomized rats were noted. Estradiol administration maintained serum levels of the steroid in estradiol-treated rats—about 10 times that of intact animals and more than 20 times that of ovariectomized animals. Morphologically, live cell counts in estradiol-treated rats were significantly higher than in intact or ovariectomized rats. Live cell counts were also significantly higher in intact than ovariectomized rats. C-3AP positive cell counts were much higher in ovariectomized rats than in intact and estradiol-treated rats. In conclusion, proestrus levels of 17β-estradiol protect hippocampal CA1 neurons against transient global ischemia, through mechanisms that appear to involve improvement of perfusion and inhibition of caspase-3 activity.

Keywords: Caspase-3; Cerebral blood flow; Histology; Proestrus estradiol; Rats; Transient global ischemia

1. Introduction

Clinical observations indicate that estrogens may be naturally occurring neuroprotectants. Extensive experimental evidence indicates that estrogens are neuroprotective against both focal and forebrain ischemia [1–13]. One critical consideration concerning estrogen replacement therapy is the effective dose range. Some investigations suggest a narrow therapeutic dose range within physiological levels [8,13], while the others indicate that high doses were necessary for estrogen to exert the neuroprotective effects against oxidative stress and/or excitatory amino acids [14–16].

Another important issue concerning estrogen replacement therapy is the mechanism(s) by which these compounds act against ischemia. Several studies demonstrated that estrogen does not change cerebral perfusion during focal cerebral ischemia [5,8,17] despite significantly reducing infarct volume; others, however, using the forebrain or global ischemia models, indicate that estrogen administration does improve CBF [13,18]. None of studies clarify histologic outcome relative to CBF or plasma estradiol concentration. Experimentally induced transient global ischemia causes selective, delayed neuron death in the hippocampal CA1 region [19–21], a phenomenon attributable to programmed cell death or apoptosis [22,23]. Whether apoptotic mechanisms play a role concerning the neuroprotective effect of estrogen against cerebral ischemia in vivo remains uncertain.

Caspase-3 protein is present at low levels in the adult brain [24,25]. Expression of both caspase-3 mRNA and protein in the brain is markedly up-regulated in response to

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ischemia [26]. In addition, ischemia induces proteolytic cleavage of the precursor protein into two subunits, one of which contains the catalytic site, i.e. caspase-3 active. Several lines of evidence indicate that the active caspase-3 serves as a key effector of the apoptotic process, contributing wholly or partially to the proteolytic cleavage of many critical proteins involved in apoptosis. Caspase-3 knock-out mice appear to lack programmed neuron death [27]. Inhibition of caspase-3 leads to a reduction of apoptotic neuron death induced by cerebral ischemia [26]. Therefore, investigation of caspase-3 activation pathway may provide an important insight regarding apoptotic mechanisms and estrogen-related neuroprotection.

In the present study, we examined whether proestrus estradiol levels could protect hippocampal CA1 neurons in rats subjected to transient global ischemia, and correlated neuron death with ending (96 h following ischemia) serum 17β-estradiol concentrations and cell death relative to caspase-3 activity.

2. Materials and methods

2.1. Animals

The experimental protocols were approved by the University of Florida Institutional Animal Care and Use Committee. Sprague–Dawley female rats weighing 225–250 g were purchased from Charles River Laboratories (Wilmington, MA). Animals were housed in pairs and acclimated with a laboratory diet and tap water ad libitum under a fixed light–dark cycle for 1–2 weeks before experimentation. A total of 53 animals were divided into non-ischemia (N), vehicle-treated (V), estrogen-treated (E) and intact (I) groups. The N ($n=3$), V ($n=16$), and E ($n=18$) groups were subjected to bilateral ovariectomies 1–2 weeks before the induction of cerebral ischemia, while the I group ($n=16$) received a sham-operative ovariectomy.

2.2. 17β-Estradiol administration and concentration determination

Estradiol was administered subcutaneously by implanting two 5-mm steroid containing silicone elastomer pellets (4 mg/ml) 24 h before ischemia onset in the E group. Corn oil pellets were implanted in the V, I and N groups. Two-pellet-implantation was selected because one estradiol pellet did not maintain serum concentration up to or above proestrus level for the duration of the experiment. The ending serum 17β-estradiol concentration was measured 96 h following the onset of cerebral ischemia and just prior to sacrifice. Blood samples were taken via the jugular vein, and the serum was separated from blood cells by centrifugation and stored frozen (−20 °C). Serum estradiol concentration was determined using duplicate serum aliquots in an ultrasensitive estradiol radioimmunoassay kit (Diagnostic Systems Lab, Webster, TX, USA).

2.3. Cerebral ischemia production

Transient incomplete global ischemia was produced as described previously [28]. Briefly, through a midline occipital-suboccipital incision, both vertebral arteries (VA) were exposed between the first and second cervical vertebra and cauterized under operating microscope magnification using a digital bipolar cauterizer. Both common carotid arteries (CCA) were occluded for 20 min in the E, I and V groups through an anterior cervical incision 24 h after the VA occlusion. All surgical procedures except for CCA occlusion were performed in the N group. Body temperature was monitored and maintained at 36.5–37.5 °C using a heating lamp during surgery, ischemia and early reperfusion time. All rats were allowed to survive 96 h following the onset of cerebral ischemia.

2.4. Arterial physiological parameter determination

The animals were anesthetized using 2.5% halothane in air supplemented with 10% pure oxygen delivered via a face-mask. Once the carotid arteries were temporarily occluded, the halothane was discontinued for the first 5 min of global ischemia, and then re-adjusted to approximately 1% in air (or less) during the last 15 min of global ischemia. Ten rats (3–4 animals in each of the V, I and E groups) were used to determine the mean arterial blood pressure (MABP), pH, pO_2, pCO_2, hematocrit, sodium, potassium and calcium. One femoral artery was cannulated and blood samples were taken before and 15 min following the onset of ischemia. MABP was monitored using a pressure transducer (Harvard Apparatus, Holliston, MA, USA) and blood parameters were determined using i-STAT portable clinical analyzer (Abbott Laboratories, East Windsor, NJ, USA).

2.5. Local cerebral blood flow measurement

Cerebral blood flow (CBF) was measured in 22 rats (E group, $n=8$; I group, $n=7$; V group, $n=7$) using the hydrogen clearance method (the Digital UH meters of Type MHG-D1, Unique Medical, LDT, Chifu-shi, Tokyo, Japan) as described previously [29,30]. Animals were anesthetized by intraperitoneal ketamine (60 mg/kg) and xylazine (10 mg/kg) injection, and the head was fixed in a stereotaxic frame. One teflon-coated platinum electrode was stereotactically inserted to a depth 3 mm from the brain surface into the right hippocampus through a burr-hole placed 4.5 mm posterior to the Bregma and 2.5 mm lateral to the midline. A second electrode was inserted to a depth 1 mm from the brain surface into the left parietal cortex through a burr-hole 2 mm posterior to the Bregma and 2 mm lateral to the midline (Fig. 1). CBF was

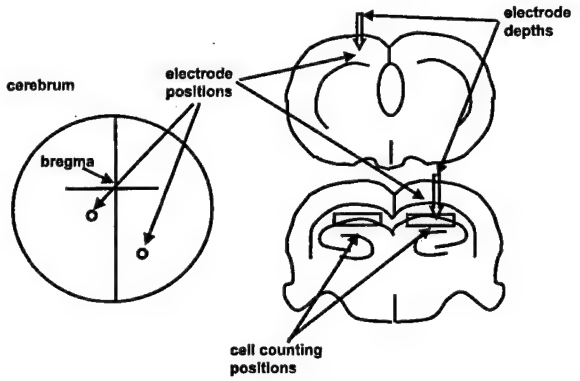

Fig. 1. Schematic illustration showing the positions for electrode insertion and hippocampal live cell and immunofluorescent positive cell counts. Two electrodes were simultaneously inserted into the left parietal cortex and right hippocampus for determining local cerebral blood flow. The live cell and caspase-3 positive peptide positive cell counts were performed in the middle hippocampal CA1 sector.

measured 10 min before ischemia, 10 min into the ischemia, and 10 and 30 min after reperfusion was re-established.

2.6. Histologic and immunofluorescent assessments

Twenty-one rats ($n=6$ in each of the E, I, and V groups and $n=3$ in the N group) were used for histologic and immunofluorescent assessments. The anesthetic protocol was identical to that used for determining the arterial physiological parameters. After survival for 96 h, the animals received intraperitoneal ketamine (60 mg/kg) and xylazine (10 mg/kg), and intra-arterial perfusion with 100 ml of saline followed by 100 ml of 4% buffered paraformaldehyde phosphate.

A 3 mm-thick tissue block 5.5–8.5 mm from the frontal pole was taken from each brain and coronally sectioned and paraffin-embedded. Four 5 μm-thick slices were cut from each block, each containing a cross-section of the dorsal hippocampus. Adjacent 5-μm slices were evaluated using hematoxylin and eosin (H&E) staining and immunofluorescent staining of caspase-3 active peptide (C-3AP).

The sections were deparaffinized in xylene, rehydrated through graded ethanol, and washed with phosphate buffered solution (PBS)-Tween 20. After incubation in 4% normal goat serum diluted by PBS-Tween 20, the slides were incubated overnight at 4 °C with C-3AP rabbit antibody (R&D Systems, Minneapolis, MN, USA) 1:200 diluted in 4% normal goat serum PBS-Tween 20. After incubation with Alexa Flur™ 488 goat anti-rabbit IgG (1:200) for 75 min, the slides were stained using 100 μM DAPI. Sections were examined under fluorescence optics using excitation and barrier filters appropriate for selectively visualizing FITC and DAPI, respectively. A rabbit isotype control IgG (ZYMED Laboratories, South San Francisco, CA, USA) was used to replace the first antibody as a negative control. In processing the staining in the V group, samples from group N were included as a further negative control.

Live cell and C-3AP positive cell counts were performed on the middle of the hippocampal CA1 sectors bilaterally (Fig. 1). The average cell number in each hippocampal CA1 sector was derived from five view fields under a 400× microscope magnification lens using a 10 × 10-mm grid. The mean cell number was generated by averaging the cell counts from both sides of the hippocampus. The live cell ratio was used as a histologic measurement of viability. This ratio was produced by dividing the mean cell number of live cells in the V, I and E groups by that in the N group.

Histologic and immunofluorescent assessments were not performed in the same rats used for CBF and arterial blood

Table 1
Experimental protocol and its complement

Group	Experimental rat number	Anesthetic agents	Ovariectomy	BVAO	BCAO	Serum estradiol	H&E staining	C3AP staining
For pathology (n = 21)								
Estradiol	6	Halothane	+	+	+	6	6	3
Vehicle	6		+	+	+	6	6	3
Intact	6		Sham-operation	+	+	6	6	3
No ischemia	3		+	+	Sham-operation	3	3	3
For physiological parameters (n = 10)								
Estradiol	4	Halothane	+	+	+	–	–	–
Vehicle	3		+	+	+	–	–	–
Intact	3		Sham-operation	+	+	–	–	–
For cerebral blood flow measurement (n = 22)[a]								
Estradiol	8	Ketamine + xylazine	+	+	+	–	–	–
Vehicle	7		+	+	+	–	–	–
Intact	7		Sham-operation	+	+	–	–	–

BVAO, bilateral vertebral artery occlusion; BCAO, bilateral carotid artery occlusion; H&E, hematoxylin and eosin; C3AP, caspase-3 active peptide.

[a] 10 rats, 4 in estradiol group and 3 in each of vehicle and intact groups, respectively, were excluded for analysis of cerebral blood flow because of sudden death or respiration stop occurring 3–6 min following BCAO.

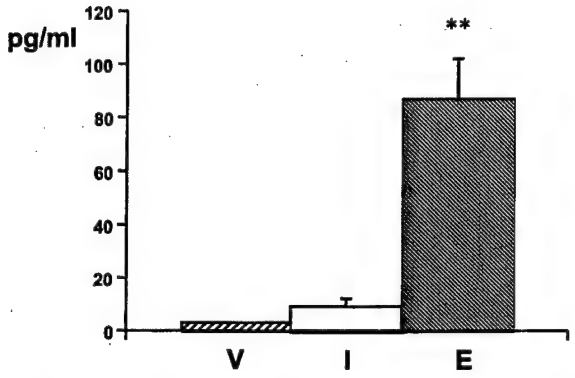

Fig. 2. Ending serum 17β-estradiol concentrations. Eighteen female rats were divided into ovary intact (I), vehicle (V) and estradiol (E) treated groups ($n=6$ each, see Materials and methods for details). Blood samples were obtained just before sacrifice to correlate the serum 17β-estradiol concentrations with histological and immunofluorescent outcome. $^{**}p<0.01$ vs. the V and the I groups.

parameter determination. The former showed surgical damage due to electrode insertion, and the latter were administered heparinized saline to keep the arterial cannula open and to compensate for blood loss due to sampling. These treatments might have complicated the immunofluorescent results.

2.7. Statistical analysis

Fisher's ANOVA was used for comparison of blood physiological parameters, blood pressure, cerebral blood flow, 17β-estradiol concentration, the live cell ratio and C-3AP positive cell counts between groups. The statistical software Statview-J 4.1 (Abacus Concepts) was used. A p value <0.05 was considered significant.

3. Results

The experimental protocol and its components are documented in Table 1. Three rats in both the I and V groups and four rats in the E group stopped spontaneous respiration between 3 and 6 min following global ischemia onset, each of which was anesthetized with ketamine and xylazine. These animals were to be used for measuring CBF changes, and all (total $n=10$) were excluded from further analysis. No rats stopped breath or died before the histologic assessment when the halothane-anesthetic protocol was employed.

3.1. Physiological parameters

No significant differences in arterial blood pressure, pH, pO_2, pCO_2, hematocrit, sodium, potassium and calcium were noted between the V, I and E groups before the onset of ischemia (data not show). The MABP in all groups increased by 48–53 mm Hg by 15 min following ischemia. Cerebral ischemia-related hyperventilation and halothane cessation followed by reinstitution of a reduced halothane supply resulted in an increased arterial pH and decreased pCO_2 at 15 min following ischemia, with no significant differences between groups.

3.2. Estrogen levels

The ending serum concentrations of 17β-estradiol were 3 ± 0 and 9 ± 3 pg/ml in the V and I groups, respectively (Fig. 2). The concentration in the N group was similar to that in the V group (3 ± 0 pg/ml). Estradiol administration raised the steroid levels to 87 ± 15 pg/ml, about a

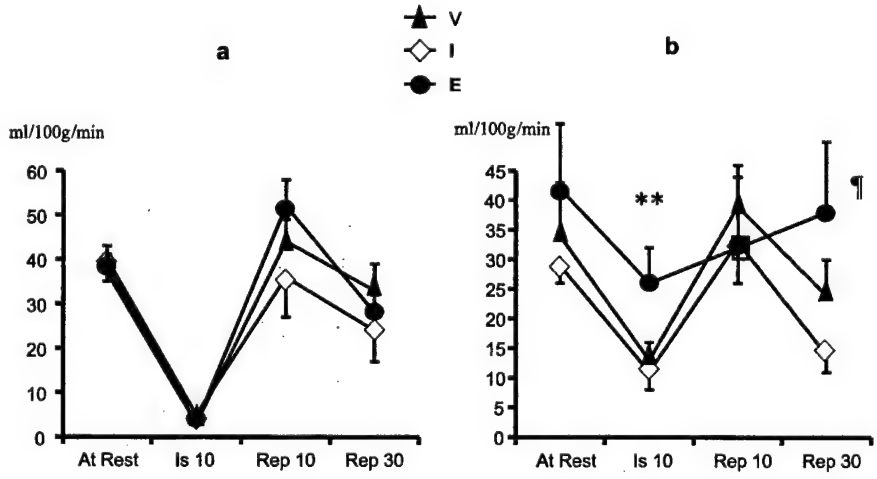

Fig. 3. Changes in cerebral blood flow in the parietal cortex (a) and hippocampus (b) following transient global ischemia. The animals were divided into ovary intact (I), vehicle (V) and estradiol (E) treated groups. Local cerebral blood flow was determined before, during (10 min into the ischemic interval), and after transient global cerebral ischemia (10 and 30 min after perfusion was reestablished) using hydrogen clearance method. $^{**}p<0.01$ vs. the V and the I groups; ¶$p<0.05$ vs. the I group.

10-fold increase over that of intact rats and more than 20-fold that of ovariectomized rats.

3.3. Changes in CBF

CBF in the parietal cortex was 37–40 ml/100 g/min in the V, I and E groups before the onset of cerebral ischemia (Fig. 3a). Global ischemia resulted in decreased CBF to 7–11% of the pre-ischemia resting state. Following reperfusion, the CBF initially recovered to close to or higher than the resting state at 10 min, but then dropped to 60–87% of the resting levels in all groups. No significant CBF differences in the parietal cortex were noted at any time between groups.

Hippocampal CBF ranged from 29 to 42 ml/100 g/min, with no significant differences between groups before the onset of cerebral ischemia (Fig. 3b). Marked CBF reduction was noted following ischemia onset and 30 min after circulation was re-established in the V and I groups but not in the E group. The CBF in the E group was significantly higher than in the V and I groups during the ischemia, and was higher than the I group after 30 min of reperfusion. No significant CBF differences between the I and the V groups were noted at any time.

3.4. Histologic and immunofluorescent assessments

Twenty minutes of four-vessel occlusion induced severe cell loss in the V group (Fig. 4A–D), although the damage was not homogeneously distributed on both sides. Five of six rats in the V group showed much more severe damage in the medial and middle sectors of the hippocampal CA1 region on one side and in the medial sector on the opposite side. Damage to the middle sector on the opposite side and

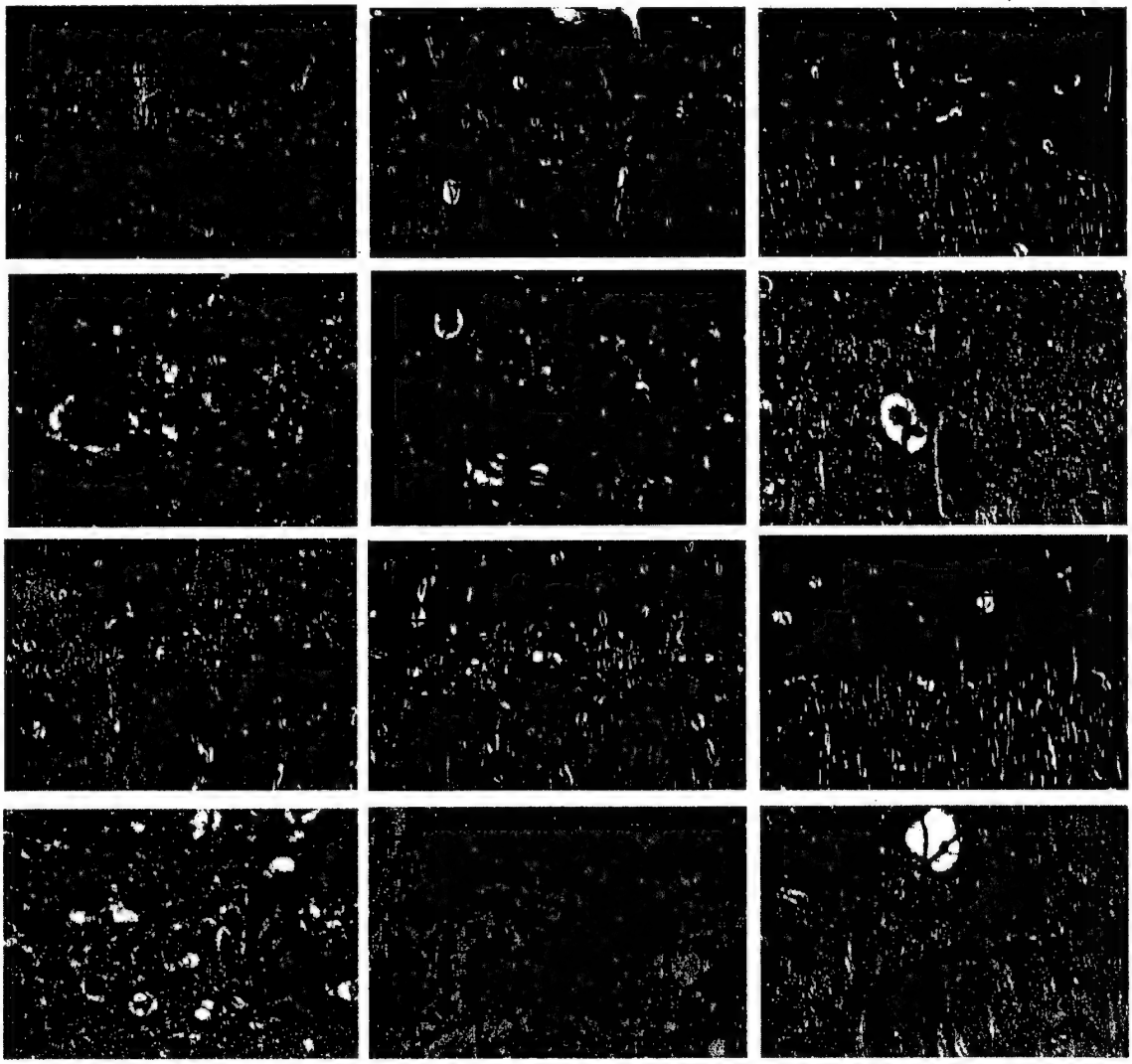

Fig. 4. Representative histology of the middle hippocampal CA1 sector following transient global ischemia. The images were obtained from two vehicle-treated (A–D), two ovary intact (E–H) and two estradiol-treated (I–L) rats. The slices were stained using hematoxylin and eosin. Original magnification is 100 × for (A), (C), (E), (G), (I), and (K). Images (B), (D), (F), (H), (J), and (L) show those noted in the squares of (A), (C), (E), (G), (I), and (K), respectively, at 400 × magnification.

both lateral sectors was rather mild. Four rats displayed severe cell loss on the right side and only one on the left.

Two of the six rats in the I group exhibited similar damage as that observed in the V group (non-homogeneous severe cell loss in the CA1 region limited to one side). The other animals in this group exhibited clustered cell loss as shown in Fig. 4E–H. 17β-estradiol provided robust protection. None of the six rats in the E group showed severe damage in any sectors of the hippocampal CA1 region, and most exhibited only spotty cell loss (Fig. 4I–L).

Immunofluorescent images on slices adjacent to those used for histologic analysis demonstrated that the morphology of C-3AP positive cells and their numbers paralleled the severity of the histologic ischemic damage. In the V group, the C-3AP immunoreactivity was primarily localized in the deformed nuclei (Fig. 5C and F). In the I group, the immunostaining was often observed in both the disfigured nuclei and the cytoplasmic compartment (Fig. 5E). Some cells in the E group also exhibited mixed nuclear-cytoplasmic compartment immunostaining (Fig. 5D), although the nucleic deformation labeled with C-3AP immunoreactivity was rather mild in the estrogen-treated rats.

The histologic and immunofluorescent assessments are summarized in Fig. 6. The live cell ratio, calculated in reference to the N group, was $35 \pm 3\%$ in the V group, a

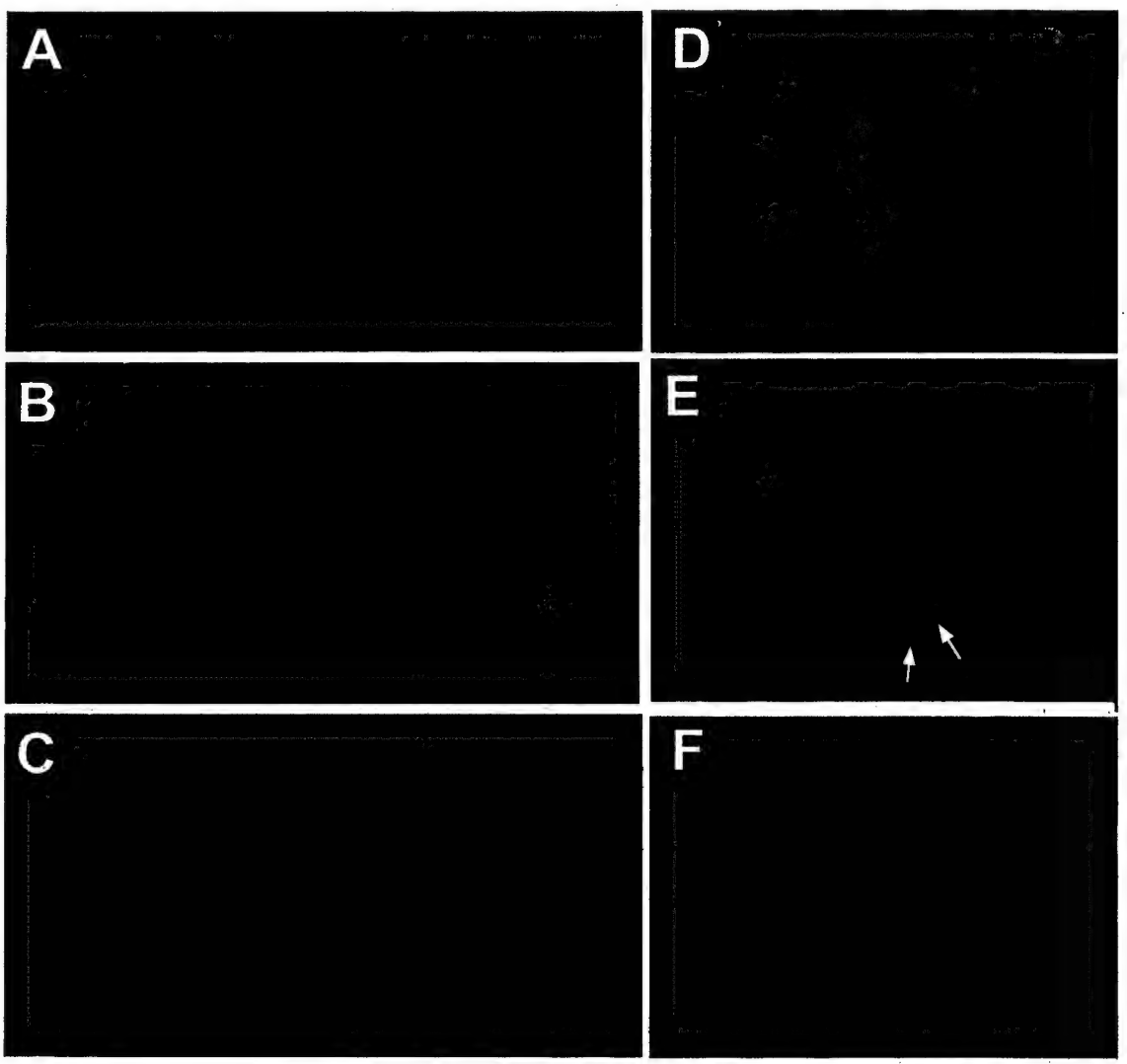

Fig. 5. Representative images of caspase-3 active peptide (C-3AP) positive cells in the middle hippocampal CA1 sector. (A) Immunofluorescent staining in a no-ischemic rat. Little C-3AP positive cells were observed. (B) The staining using a rabbit isotype control IgG to replace the first antibody against C-3AP processed in a vehicle-treated rat. Little specific staining was observed. (C) The staining using a brain slice adjacent to the slice shown in (B). C-3AP positive immunoreactivity stains green, while the nuclei double-stained with DAPI appear blue. (D) The staining in an estradiol treated rat. Some cells exhibited mixed C-3AP nuclear-cytoplasmic compartment immunostaining (arrow-pointed). The nucleic deformation labeled with C-3AP immunoreactivity was rather mild. (E) The staining in an ovary-intact rat. The immunoreactivity was often observed in both the disfigured nuclei and the cytoplasmic compartment (arrow-pointed). (F) A high magnificence of figure (C). The C-3AP immunoreactivity was primarily localized in the deformed nuclei (arrow-pointed). Original magnification is 200 × for (A), (C), and (E), and 630 × for (B), (D), and (F).

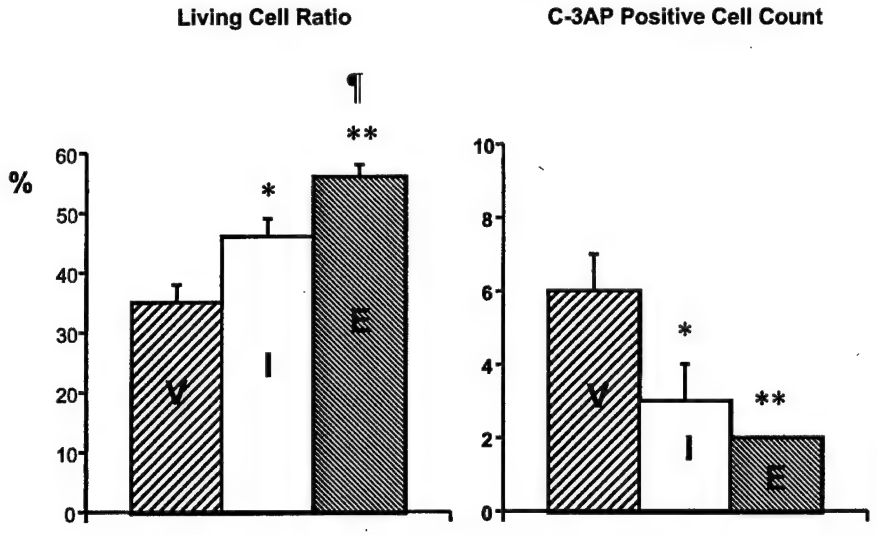

Fig. 6. Comparison of live cell and caspase-3 active peptide positive cell counts between vehicle-treated (V), ovary intact (I), and estradiol-treated (E) rats. The live cell ratio was produced by the mean live cell number in V, I and E groups divided by that in the no-ischemic group, respectively (see text for details). $*p<0.05$ vs. the V group, $**p<0.01$ vs. the V group; $¶p<0.05$ vs. the I group.

value which was significantly lower than that of both in the I ($46 \pm 3\%$, $p<0.05$) and the E groups ($56 \pm 2\%$, $p<0.001$). The ratio was also lower in the I group compared to the E group ($p<0.05$). The C-3AP positive cell number was significantly higher in the V group than in the I and E groups ($p<0.05$ and $p<0.01$, respectively).

4. Discussion

The present study demonstrates that proestrus levels of 17β-estradiol ameliorate ischemic damage in the hippocampal CA1 region of rats subjected to transient global ischemia. A higher neuron survival rate in the estrogen-treated rats as compared with that in rats with or without bilateral ovariectomies was accompanied by the improvement of CBF and the reduction of C-3AP positive cell numbers. These neuroprotective effects may be linked to both perfusion-dependent and perfusion-independent mechanisms. The neuroprotective effects exhibited in ovary-intact rats were much weaker than in the estrogen-replaced group, perhaps because the estradiol concentration and the CBF level were approximately close to those in the estrogen-depleted group.

Physiological plasma 17β-estradiol levels in rats range between 10 and 30 pg/ml, and may increase up to 80–140 pg/ml during the proestrus period [31–33]. Pellet implantation leads to an immediate increase of the serial 17β-estradiol concentration, reaching the peak 1 h following the implantation in ovariectomized, non-ischemic rats [34]. One pellet implanted 24 h before the production of cerebral ischemia provided slow release of the steroid at least for 8 days, maintaining the concentrations of 92 ± 12, 35 ± 10 and 37 ± 7 pg/ml at 72, 120 and 192 h, respectively, and these levels only delayed neuron death [35]. To approach those levels seen during the proestrus period, we used two-pellet implantation in the present study. The neuron-sparing effects demonstrated in this study extend our previous report, showing that following transient global ischemia, proestrus estrogen levels provide substantial neuroprotection. Some neuroprotective effects were also seen in intact rats in which the ending serum 17β-estradiol paralleled the metestrus level. The discrepancy may be ascribed to a synergetic effect provided by other ovarian hormones. For example, progesterone has membrane stabilizing effect that may reduce the damage caused by lipid peroxidation [36], and may ameliorate the ischemic damage following the middle cerebral artery occlusion [37,38].

Pelligrino et al. [13] argued against any neuroprotection with supraphysiological estrogen levels. Discrepancies in estrogen dosage and models limit the direct comparison of our data with theirs. Mean arterial blood pressure was subjectively controlled in their study (unilateral right carotid occlusion with hemorrhagic hypotension) but not in ours. In addition, their model might involve anti-coagulating agents gaining access to the ischemic area when cerebral circulation is re-established by recirculating and transfusing the blood back into the carotid artery. Their death rate was as high as 32–67% [13] but zero in our histologic assessment group; such disparate survival rates raise the possibility that some other undefined bias has been introduced between groups within these studies.

The hydrogen clearance method provides absolute measurements of local CBF. The CBF in rats anesthetized with katamine and xylazine was similar to those anesthetized with amobarbital in our previous studies [29,30]. The anesthetic route and agents were selected because we already had extensive experience using them for both focal and global ischemic models [1,9,35], and also because the method facilitates stereotaxic frame fixation to insert the

CBF measuring electrodes. On the other hand, 43–50% of rats in each group using katamine and xylazine stopped spontaneous respiration between 3 and 6 min following bilateral CCA occlusion. Inhibition of the respiratory control center in the brain stem due to both global ischemia and the anesthetics may account for this phenomenon. Nevertheless, our observed reduction in parietal cortex CBF following global ischemia was consisted with those using laser-Doppler flow meter reported by Pelligrino et al. [13].

The reduction of hippocampal CBF during bilateral CCA occlusion was very profound in our study (decreased by 60–62% in the V and I groups) compared with those using the model of Pulsinelli et al. [20], in which the CBF in the hippocampus decreased by only 42% [39]. Estrogen administration improved CBF during the occlusion in the hippocampus but not in the parietal cortex. During the first 30 min of reperfusion, the parietal CBF showed biphasic changes (hyperperfusion followed by hypoperfusion) in all three occlusion groups. Similar changes were noted in the hippocampal flow in the V and I groups, but not in estrogen-treated rats, where the CBF gradually recovered to the level similar to that prior to the ischemia. A much higher serum level of 17β-estradiol in estrogen-treated rats as compared to that in other two groups is presumably responsible for these differences.

Hippocampal neurons undergo delayed death following transient global ischemia, providing a potential in vivo apoptotic cell death model. The key elements of apoptosis include chromatin condensation, DNA fragmentation, caspase activation or processing, and selective substrate cleavage. Our study was consistent with the report conducted by Chen et al. [26] by showing that at least some of the neurons in the hippocampal CA1 region died through a caspase-3 activating pathway. The novel finding in our study is that the attenuation of ischemic damage in the hippocampal CA1 region due to 17β-estradiol administration was associated with reduction of the C-3AP positive cell numbers.

Chen et al.'s [26] study also indicated that caspase-3 immunoreactivity in the hippocampal CA1 region increased in the neuron cytoplasm at 8–24 h, followed by both cytoplasmic and nuclear localization at 72 h following global ischemia. Permanent focal cerebral ischemia appears to accelerate the shift from cytoplasmic to nuclear localization within 24 h [40]. In our study, the C-3AP positive staining was primarily localized to the nucleus in ovariectomized rats, while a mixed nuclear-cytoplasmic compartmentalization was often observed in ovary-intact and estrogen-treated rats, indicating that estrogen might modify or delay the translocation.

Our study did not assess how estrogens affect caspase-3 activation. In addition to perfusion-dependent effects, estrogen inhibited caspase-3 activity in cardiac myocytes induced by staurosporine but not basal caspase-3 activity [41], indicating that the steroid may affect caspase-3 activation through an indirect pathway. Estrogen may up-regulate anti-apoptotic factor Bcl-2 [7], which in turn suppresses cytochrome c release and the ensuing caspase-3 activation. Estrogen may also suppress intracellular oxygen radicals and exert neuroprotection against oxidative stress, specially when high concentration of estradiol is used [9,42]. A recent study suggests that the anti-apoptotic neuroprotective effects of estrogen may be mediated by transcription through the activator protein-1 site downstream from c-Jun NH(2)-terminal kinase and caspase-3 activation [43]. These mechanisms may collectively account for the different intercellular localization of C-3AP immunoreactivity and differential live cell counts in the hippocampal CA1 neurons between ovariectomized, ovary-intact, and estradiol-treated rats.

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Appendix I

Protective Effect of Estrogens Against Oxidative Damage to Heart and Skeletal Muscle *In Vivo* and *In Vitro* (44463)

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Abstract. Estrogen has been shown to protect skeletal muscle from damage and to exert antioxidant properties. The purpose of the present study was to investigate the antioxidant and protective properties of estrogens in rodent cardiac and skeletal muscle and H9c2 cells. Female Sprague-Dawley rats were separated into three groups, ovariectomized (OVX), ovariectomized with estrogen replacement (OVX + E2), and intact control (SHAM), and were assessed at two time periods, 4 and 8 weeks. Rodents hearts were analyzed for basal and iron-stimulated lipid peroxidation in the absence and presence of β-estradiol (βE2) by measuring thiobarbituric acid reactive species (TBARS). Isolated soleus (SOL) and extensor digitorum longus (EDL) were analyzed for creatine kinase (CK) efflux. Using H9c2 cells, the *in vitro* effects of βE2 and its isomer α-estradiol were investigated under glucose-free/hypoxic conditions. TBARS assay was also performed on the H9c2 in the presence or absence of βE2. The results indicate that OVX rodent hearts are more susceptible to lipid peroxidation than OVX + E2 hearts. OVX soleus showed higher cumulative efflux of CK than OVX + E2. Furthermore, H9c2 survival during oxidative stress was enhanced when estrogen was present, and both OVX hearts at 4 weeks and H9c2 cells particularly were protected from oxidative damage by estrogens. We conclude that estrogen protects both skeletal and cardiac muscle from damage, and its antioxidant activity can contribute to this protection.

[P.S.E.B.M. 2000, Vol 223]

The importance of estrogen in protecting tissues is currently receiving increasing attention. Estrogen is a fat-soluble hormone that can contribute to membrane fluidity by direct interactions with phospholipids. It has also been suggested that estrogen can suppress free radical–induced peroxidation chain reactions because of the similarity in structure to vitamin E, namely the presence of the hydroxyl group on the phenolic A ring (1, 2). By the age of 55, women normally pass through menopause resulting in ovarian exhaustion of follicles and a precipitous decline in ovarian steroids. Women can live more than a third of their lives in an estrogen-deficient state; however, overwhelming evidence suggests that postmenopausal estrogen loss can have negative effects on the brain (3, 4), bone (5, 6) and cardiovascular system (7). Despite this evidence, less than 25% of postmenopausal women receive estrogen replacement therapy (ERT) largely because of the fear that ERT increases the risk of uterine cancer and perhaps mammary tumors.

The normal endogenous production of free radicals creates both beneficial and detrimental effects. Recent evidence suggests a link in the over-production of free radicals and/or decreases in antioxidant capacity with the development of disease (e.g., cancer, atherosclerosis, and Alzhei-

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mer's) (8). Likewise, it has been suggested that free radical–induced processes may occur postmenopausal, thereby stimulating research into the role of these molecules during this physiological state. One consequence of the overproduction of free radicals is lipid peroxidation and damage to membranes. Free radicals can also cause damage to protein and mitochondrial and nuclear DNA. Endogenous antioxidant systems attempt to prevent or stop free radical damaging cascades to help maintain cellular integrity. The importance of free radical production and antioxidant status is evident in the negative correlation between the production of free radicals and longevity (9). Since it is believed that estrogen can act as an antioxidant, a decrease in endogenous levels can increase free radicals, thereby potentially causing adverse effects in a variety of tissues in postmenopausal women.

Similar to the negative effects that estrogen deprivation has on the brain, bone, and cardiovascular system, estrogen deprivation could increase vulnerability of skeletal muscle to damage. The increased vulnerability may lead potentially to muscle wasting and decreased strength and can partially account for the increased incidence of falls in elderly women and a general decline in the quality of life (10–12). In addition, estrogen's role in protecting skeletal muscle has been associated with exercise-induced muscle damage by reducing serum creatine kinase levels (13) and reducing delayed-onset muscle soreness (14). Tiidus (15) assessed estrogen's role in diminishing exercise-induced muscle damage and proposed that estrogens may exert their protective effects *via* direct antioxidant or membrane stabilization actions. Like skeletal muscle, cardiac muscle is also prone to free radical–induced damage (16) and could be an additional tissue responsive to estrogens.

The objective of these studies was to investigate the antioxidant and protective properties of estrogens in rodent cardiac and skeletal muscle. Furthermore, the protective role of estrogen was investigated using the H9c2 cell line, a cell line that demonstrates both skeletal and cardiac muscle properties (17). The overall hypothesis is that estrogen deprivation increases oxidative stress resulting in lipid peroxidation in muscle cells. Conversely, estrogen supplementation can offset the toxic effects in the rodent heart, skeletal muscle, and H9c2 cell line.

Materials and Methods

Animals and Treatments. All animal protocols were approved by the University of Florida Institutional Animal Care and Use Committee in accordance with NIH guidelines. Two-week-old female Sprague-Dawley rats (Harlan, Indianapolis, IN) were divided into three groups of eight animals: 1) intact controls (SHAM); 2) ovariectomized (OVX); and 3) ovariectomized animals receiving 17β-estradiol (βE2) supplementation (OVX + E2). Bilateral ovariectomy was done using the dorsal approach as described by Singh *et al.* (18). SHAM animals underwent the same surgical procedures without the removal of the ovaries. Five-millimeter Silastic implants containing either βE2 (mixed 1:1 with cholesterol; OVX + E2 group) or cholesterol (SHAM and OVX groups) were prepared as previously described by Singh *et al.* (18) and implanted under the skin concurrent with the ovariectomy procedure. Every 2 weeks, the Silastic implants were repositioned to maintain steroid diffusion from the implant.

At 4 or 8 weeks postovariectomy, animals were anesthetized with methoxyflurane, while a vaginal lavage was performed and blood obtained *via* cardiac puncture. The animals were then decapitated, and the heart, soleus (SOL) muscles, and extensor digitorum longus (EDL) muscles were harvested. SOL and EDL muscles were analyzed immediately for *in vitro* enzyme release. Blood samples were centrifuged, and the plasma fraction stored at –80°C until analyzed for creatine kinase, progesterone, and estrogen levels.

Effects of Estrogen on Hypoxia and Glucose Deprivation in the H9c2 Cell Line. H9c2 cells (culture passages 11–15, ATCC, Rockville, MD) were maintained in DMEM media (GIBCO, Gaithersburg, MD) supplemented with 10% charcoal-stripped fetal bovine serum (Hyclone, Logan, UT) at 37°C under 10% CO_2/90% air using standard culture techniques.

Hypoxia experiments were initiated when cells were ≈ 50% confluent in Nunc 35-mm dishes (Fisher Scientific, Orlando, FL). The medium was changed into glucose-free, serum-free DMEM with hydroxypropyl-β-cyclodextrin (HPCD) encapsulated βE2 (Sigma Chemical Co., St. Louis, MO), HPCD encapsulated 17α-estradiol (αE2; Steraloids, Wilton, NH) or HPCD as the vehicle control. Steroids were used at a final concentration of 2 n*M* or 200 n*M*. Dishes were placed immediately in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA). The chamber was flushed with 100% N_2 for 15 min to achieve hypoxic conditions, and the cells were incubated in the chamber for 12 hr at 37°C. Cells were then returned to 10% CO_2/90% air for 12 hr before viability assessment. HPCD encapsulation of αE2 has been described previously (19). Viability was assessed by exposing cells to 1 μ*M* Calcein AM and 1 μg/ml propidium iodide (Molecular Probes, Eugene, OR) in PBS (pH 7.4) for 15 min. Cells were visualized using a fluorescent Nikon microscope, and two random fields were photographed. Live cells were distinguished by the presence of a bright green fluorescence and the absence of nuclear staining by propidium iodide.

Cumulative CK Activity from Isolated Muscles. EDL and SOL muscles were placed into a Teflon-coated basket and immersed in 9 ml of carbogenated (95% O_2/5% CO_2) balanced salt solution (BSS) at pH 7.4 as described in earlier work (20). After the solutions were placed in the bath, the BSS was drained from the incubation vessels at 30-min intervals followed by the addition of fresh medium over a 4-hr period. These drained solutions at each period were analyzed for CK. Myotoxicity is calculated from the

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Appendix J

Oxidative Damage to Human Lens Epithelial Cells in Culture: Estrogen Protection of Mitochondrial Potential, ATP, and Cell Viability

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PURPOSE. Epidemiologic studies demonstrate a higher incidence of cataracts in estrogen-deprived postmenopausal women, but the mechanism for the increased risk of cataracts is unclear. An elevated level of H_2O_2 in aqueous humor and whole lenses has been associated with cataractogenesis. In the present study, for the first time, the protective effect of estrogens against oxidative stress were tested in cultured human lens epithelial cells (HLECs).

METHODS. To investigate the involvement of 17β-estradiol (17β-E_2) in protection against oxidative stress, HLECs were exposed to insult with H_2O_2 at a physiological level (100 μM) over a time course of several hours, with and without pretreatment with 17β-E_2. Cell viability was measured by calcein AM assay, and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was used to determine intracellular reactive oxygen species (ROS). Intracellular adenosine triphosphate (ATP) level was quantified with a luciferin- and luciferase-based assay and mitochondrial potential ($\Delta\Psi_m$) was monitored by a fluorescence resonance energy-transfer technique.

RESULTS. H_2O_2 caused a dose-dependent decrease in mitochondrial membrane potential, intracellular ATP levels, and cell viability. Dose-dependent increases in cell viability and intracellular ATP level were observed with pretreatment of 17β-E_2 for 2 hours before oxidative insult. At 1 nM, 17β-E_2 increased cell viability from 39% ± 4% to 75% ± 3%, and at 100 nM or higher, it increased survival to greater than 95%. The level of intracellular ATP approached normal with 17β-E_2 at 100 nM or higher. Pretreatment with 17β-E_2 did not diminish intracellular ROS accumulation after exposure to H_2O_2. Moreover, two nonfeminizing estrogens, 17α-E_2 and ent-E_2, both of which do not bind to either estrogen receptor α or β, were as effective as 17β-E_2 in the recovery of cell viability. The estrogen receptor antagonist, ICI 182,780, did not block protection by 17β-E_2. Both 17β- and 17α-E_2 moderated the collapse of $\Delta\Psi_m$ in response to either H_2O_2 or excessive Ca^{2+} loading.

CONCLUSIONS. The present study indicates that both 17α- and 17β-E_2 can preserve mitochondrial function, cell viability, and ATP levels in human lens cells during oxidative stress. Although the precise mechanism responsible for protection by the estradiols against oxidative stress remains to be determined, the ability of nonfeminizing estrogens, which do not bind to estrogen receptors, to protect against H_2O_2 toxicity indicates that this conservation is not likely to be mediated through classic estrogen receptors. (*Invest Ophthalmol Vis Sci.* 2003;44:2067–2075) DOI:10.1167/iovs.02-0841

Age-related cataracts are a leading cause of visual impairment and blindness, and an ever-increasing health problem with the aging of the world population. In the United States, approximately 1.35 million cataract surgeries are performed annually at a cost of more than $3 billion.[1] Cataract represents a large financial burden on health-care systems, and there remains a need to develop effective pharmaceuticals for the prevention or treatment of cataract.

There is a higher incidence of cataract in postmenopausal women than in age-matched men, which leads to the notion that the absence of estrogens may contribute to the increased risk.[2–6] Indeed, epidemiologic studies indicate beneficial effects of hormone replacement therapy (HRT) against cataract in postmenopausal women.[7–12] For example, the Beaver Dam Eye Study[10] and the Salisbury Eye Evaluation Project[7] have both found protective associations between hormone use and lens nuclear opacity. In addition, another large cross-sectional study, the Blue Mountains Eye Study,[8] found that HRT was associated with reduced cortical opacity in lens. Recent epidemiologic reevaluation of the Blue Mountains Eye Study determined a significant trend for increasing incidence of nuclear cataract in postmenopausal women.[11] Weintraub et al.,[12] recently evaluated HRT and lens opacities in a population of 480 postmenopausal women and determined that "current use of estrogen-only preparations was associated with a 49% decreased risk of nuclear opacities compared with never use." Studies using tissue culture and animal models also suggest beneficial effects of estrogen in lens. In a lens culture system, estrogen protected lenses against cataracts induced by transforming growth factor (TGF)-β.[13] Estrogen has also been reported to exert protective effects in a rat model of age-related cataracts induced by methylnitrosourea (MNU).[14]

Several studies have demonstrated the beneficial effects of the antioxidant activity of estrogen and, further, that the hormone's action is independent of classic receptor-dependent mechanisms. Our laboratory has shown that estradiol at physiological concentrations can block membrane oxidation.[15] Estrogen treatment has been shown to reduce lipid peroxidation induced by glutamate and further to attenuate the acceleration of intracellular peroxide production resulting from exposure to H_2O_2[16] and by mitochondrial electron transport inhibitors.[17] Consistent with these data are studies showing that estrogen inhibits formation of lipid peroxyls and oxidation of low-density lipoproteins in vitro.[18,19] In vivo studies have demonstrated that estrogen replacement therapy provided by transdermal patch reduces low-density lipoproteins.[20] These effects of estrogen do not appear to require estrogen receptors (ERs),[21–23] suggesting that estrogen exerts antioxidant activities through ER-independent mechanisms.

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In the current study, we tested, for the first time, the protective effects of estrogens against oxidative stress using in vitro cultured human lens epithelial cells (HLECs). Elevated levels of H_2O_2 are found in the lenses and aqueous humor of patients with cataract,[24-26] and it is held that H_2O_2 is a major oxidant that contributes to formation of cataract.[27] In the present study, we assessed the ability of 17β-estradiol (17β-E_2) to protect against the adverse effects of H_2O_2 on mitochondrial membrane potential ($\Delta\Psi_m$), intracellular adenosine triphosphate (ATP) levels, and cell viability in HLECs. Further, we attempted to assess the role of estrogen receptors (ERs) in this action of estrogens on lens viability by using two nonfeminizing estrogens that do not bind to ERs, 17α-estradiol (17α-E_2) and Ent-estradiol (ent-E_2), and an ER antagonist, ICI 182,780.

Material and Methods

Chemicals

17β-E_2 and 17α-E_2 were purchased from Steraloids, Inc. (Wilton, NH). ICI 182,780 was purchased from Tocris (Ellisville, MO). The complete enantiomer of 17β-E2, Ent-E_2, was synthesized by methods that we have previously described.[28]

All steroids and ICI 182,780 were dissolved in ethanol at a final concentration of 10 mM and diluted to appropriate concentration in culture medium as required. Unless otherwise stated, steroid treatment to cell cultures involved a 2-hour preincubation followed by continued administration of the steroid in the presence of H_2O_2. Those cells receiving vehicle (in place of estradiol) pretreatment were maintained in fresh culture medium at the same final ethanol concentration. Control cells were maintained in culture medium with appropriate changes of fresh medium. In experiments involving the ER antagonist ICI 182,780, it was added 30 minutes before addition of 17β-E_2.

H_2O_2 was purchased from Mallinckrodt Baker Inc. (Paris, KY). H_2O_2 was diluted with culture medium to final concentration before using. Calcein AM, 2,7-dichlorofluorescin diacetate (DCFH-DA), and ATP determination kits were purchased from Molecular Probes (Eugene, OR).

Cell Culture

HLE-B3 cells, a human epithelial cell line immortalized by simian virus (SV)-40 viral transformation,[29] were obtained from Usha Andley (Washington University School of Medicine, Department of Ophthalmology, St. Louis, MO) and cultured in Eagle's minimal essential medium (MEM) supplemented with 20% fetal bovine serum (Hyclone Laboratories, Logan, UT) and 20 μg/mL gentamicin (Sigma, St. Louis, MO) in 150-cm^2 culture flasks at 37°C and 5% CO_2 and 95% air. All experiments were performed with HLE-B3 cells between passages 18 and 25.

Measurement of Reactive Oxygen Species

The extent of cellular oxidative stress was estimated by monitoring the generation of reactive oxygen species (ROS) using the fluorescent dye DCFH-DA. Cells were plated 24 hours before initiation of the experiment at a density of 5000 cells per well in 96-well plates. Cells were loaded with DCFH-DA at a final concentration of 50 μM for 45 minutes. After incubation, DCFH-DA was removed, and cells were washed twice with 1× PBS (pH 7.4) and incubated with MEM containing 20% FBS with a bolus dose of H_2O_2 (50 and 100 μM) for 10 to 60 minutes. DCFH-DA fluorescence was determined at an excitation of 485 nm and an emission of 538 nm, by microplate-reader (model FL600; Biotek, Highland Park, VT). Values were normalized to the percentage in untreated control groups. It should be noted that, "DCFH-DA is taken up by cells and tissues, usually undergoing deacetylation by esterase enzymes. Oxidation of DCFH within cells leads to fluorescent dichlorofluorescein, which can easily be visualized (strong emission at 525 nm with excitation at 488 nm). This technique is becoming popular as a means of visualizing 'oxidative stress' in living cells. In addition to peroxidase/H_2O_2, several species cause DCFH oxidation, probably including $RO_2^{\bullet}$, $RO^{\bullet}$,$OH^{\bullet}$, HOCl, and $ONOO^-$, but not $O_2^{\bullet-}$or H_2O_2. Hence, this 'fluorescent imaging' is an assay of generalized 'oxidative stress' rather than of production of any particular oxidizing species, and it is not a direct measure of H_2O_2."[30]

Calcein AM Assay

Cells were plated 24 hours before the initiation of the experiment, at a density of 5000 cells per well in 96-well plates. Cells were exposed to two doses of H_2O_2 (50 and 100 μM) from 1 to 24 hours. After exposure to H_2O_2, cells were rinsed with 1× PBS (pH 7.4), and viability was assessed by the addition of 25 μM calcein AM, as previously described.[28] Calcein AM fluorescence was determined at an excitation of 485 nm and an emission of 538 nm with the microplate reader (FL600; Biotek). Percentage viability was calculated by normalization of all values to the H_2O_2-free control group (100%). Calcein staining was visualized by fluorescence microscope (Diaphot-300; Nikon, Tokyo, Japan), and cells were photographed for qualitative documentation. Four random fields of cells were examined, and photographs were taken of cells in 96-well plates.

Measurement of ATP Levels

Cells were plated at a density of 5×10^5 cells per well in 12-well plates. After 48 hours, cells were exposed to various doses of H_2O_2 from 15 minutes to 8 hours. Cellular ATP levels were quantified with a luciferin and luciferase-based assay.[31] Cells were washed with PBS once and lysed with ATP-releasing buffer (100 mM potassium phosphate buffer [pH 7.8]: 1% Triton X-100, 2 mM EDTA, and 1 mM dithiothreitol [DTT]). Ten microliters of the lysate was added to 96-well plates (InterMed, Naperville, IL) . ATP concentrations in lysate were quantified using an ATP-determination kit according to the manufacturer's instruction. The 96-well plates were then read (SpectraMAX GeminiXS plate reader; Molecular Devices, Sunnyvale, CA). A standard curve was generated with solutions of known ATP concentrations. Protein concentration of samples were determined by Bradford assay.[32] ATP levels were calculated as nanomolar ATP per milligram protein and normalized to levels in untreated control cultures.

Monitoring $\Delta\Psi_m$

$\Delta\Psi_m$ was recorded in intact and digitonin-permeabilized cells with an assay based on fluorescence resonance energy transfer (FRET) between two dyes: nonyl acridine orange (NAO; Molecular Probes), which stains cardiolipin, lipid found exclusively in the mitochondrial inner membrane, and tetramethylrhodamine (TMRE; Molecular Probes), a potentiometric dye taken up by mitochondria in accord with Nernstian dictates potential and concentration. The presence of TMRE quenches NAO emission in proportion to $\Delta\Psi_m$, whereas loss of $\Delta\Psi_m$ with consequent efflux of TMRE dequenches NAO.[33,34] The high specificity of NAO staining; selective monitoring of the fluorescence emitted by NAO, not TMRE; and the stringent requirement for colocalization of both dyes within the mitochondrion, all act in concert to allow the FRET assay to report $\Delta\Psi_m$, unconfounded by background signal arising from potentiometric dye responding to plasma membrane potential.

Twenty-four hours before assay, cells were trypsinized and plated in clear-bottomed, black-walled, 96-well plates (Costar 3606; Corning International, Corning, NY). Cells were plated at 60,000 per well for use in high-throughput screening protocols, as described previously.[16,28]

Statistical Analyses

Effects on $\Delta\Psi_m$ were quantified by calculating the area under the curve (AUC) after either Ca^{2+} challenge or addition of H_2O_2. To partially correct for variations in cell density, staining and optical aberrations in these plates, all wells were normalized to the initial relative fluorescence units (RFU) reading in each well using a fluorescence-imaging

plate reader (FLIPR; with accompanying software; Molecular Devices). In a variation of the analysis, the AUC was divided by the amount of initial quenching of NAO, which is an alternate technique to compensate for variability in cell density and staining and for optical aberrations. Dose-response data were fitted on computer by nonlinear regression analysis (sigmoid equations; Prism, ver. 3.00 for Windows; GraphPad Software, San Diego, CA). One way ANOVA and Bonferroni post hoc testing were performed with the same software.

The significance of differences among groups was determined by one-way ANOVA. Planned comparisons between groups were determined by the Tukey test. For all tests, $P < 0.05$ was considered significant.

Results

Effects of H_2O_2

ROS Accumulation. As shown in Figure 1a and as measured by DCF fluorescence intensity, exposure to H_2O_2 caused an increase in intracellular ROS accumulation in the cultured HLECs. By 60 minutes after administration, 50 μM H_2O_2 initiated a modest but linear increase in ROS content (172% ± 11%) over control cells. A higher dose of H_2O_2 (100 μM) prompted a biphasic accumulation of ROS in the cultured cells. After 40 minutes, there was an ROS buildup resulting in a moderate intracellular augmentation by 40 minutes (200% of control), but accumulation was amplified to approximately 500% of control by 60 minutes.

Intracellular ATP Content. Administration of both 50 and 100 μM H_2O_2 triggered a rapid decrease in intracellular ATP levels (Fig. 1b). Within 60 minutes, 50 and 100 μM H_2O_2 reduced ATP by 68% ± 8% and 50% ± 5% of control, respectively. Whereas the ATP levels appeared to slightly recover 2 hours after administration of H_2O_2, the overall trend was for ATP levels to decline at either H_2O_2 dose to approximately 40% of control over the remainder of the time course.

Cell Viability. H_2O_2 induced a time- and dose-dependent decline in cell viability (Fig. 1c). At 50 μM, it decreased cell viability to 65% ± 2% of control by 12 hours after exposure. Exposure to 100 μM H_2O_2 had a significantly greater effect on viability of HLECs, with the surviving of cells declining to 45% ± 4% by 2 hours and almost complete cell death by 8 hours after H_2O_2 treatment.

Effects of 17β-E_2 against H_2O_2 Exposure on HLECs

Because 100 μM H_2O_2 had a significant impact on ROS accumulation, intracellular ATP content, and cell viability, this concentration was selected for further studies with estrogens.

H_2O_2-Induced ROS Increase in HLECs. As in Figure 1a, 100 μM H_2O_2 progressively and significantly increased intracellular ROS over the 60-minute observation period. Concentrations of 17β-E_2 ranging from 1 nM to 10 μM did not modify intracellular accumulation of ROS (Fig. 2).

H_2O_2-Induced ATP Decline in HLECs. 17β-E_2 was examined for its protective capacity against the decline of intracellular ATP caused by exposure of cultured HLECs to exogenous H_2O_2 (Fig. 3). A 2-hour pretreatment with 17β-E_2 reversed the decline of intracellular ATP brought about by H_2O_2 in a dose-dependent manner. Whereas a low concentration of 17β-E_2 (10 nM) did not alleviate the ATP loss in response to H_2O_2 treatment, ATP levels were restored from 75% ± 4% (peroxide treatment) to 93% ± 9% of control with 100 nM 17β-E_2. At concentrations above 1 μM, 17β-E_2 completely normalized the intracellular ATP pool.

H_2O_2-Induced Cell Death in HLECs. 17β-E_2 protected against HLE-B3 cell loss due to H_2O_2 toxicity in a dose-dependent manner. H_2O_2 (100 μM) resulted in a 61% ± 4% decline in cell survival. A 2-hour preincubation with 17β-E_2 (1 nM) increased cell survival from 39% ± 4% to 75% ± 3%. At 100 nM and higher, 17β-E_2 completely protected against H_2O_2 toxicity (Fig. 4).

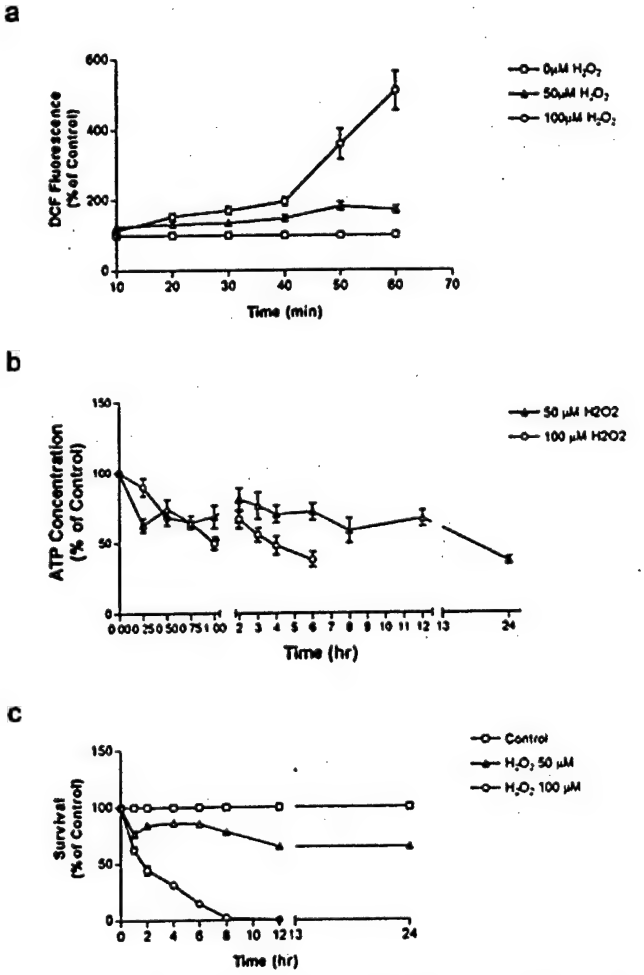

FIGURE 1. (a) Effects of H_2O_2 on ROS accumulation in HLECs. The control group was not treated with H_2O_2 after loading DCFH-DA. Depicted mean ± SEM (n = 8) percentages of DCF fluorescence normalized to the control (no H_2O_2) at each sampling time. (b) Time course of the effects of H_2O_2 (50 and 100 μM) on intracellular ATP levels in HLE-B3 cells. Data are expressed as a percentage of control levels (non-H_2O_2-treated cells at each sampling time) and represent the mean ± SEM of determinations made in four to six cultures per group. (c) Time course of the effects of H_2O_2 on viability of HLE-B3 cells. Data are expressed as a percentage of control group (non-H_2O_2-treated cells at each sampling time) and represent the mean ± SEM (n = 8). In all panels, when SEM bars are not shown, they are obscured by the symbol.

Effects of 17α- and 17β-E_2 on Ca^{2+}- and H_2O_2-Mediated Collapse of $\Delta\Psi_m$

As is the case with other cell types,[33,34] acute increases in cytosolic Ca^{2+} induced through the ionophore ionomycin caused a dose-dependent collapse of $\Delta\Psi_m$ in intact HLECs (Fig. 5). Even a brief 5-minute incubation with either 17α- or 17β-E_2 at 0.5 μM substantially reduced the magnitude of this ionomycin-induced $\Delta\Psi_m$ collapse (Fig. 5), reflected by an increase in EC_{50} from 0.95 μM to 1.6 and 2.3 μM for 17α- and 17β-E_2, respectively. Thus, it required more Ca^{2+} to induce compara-

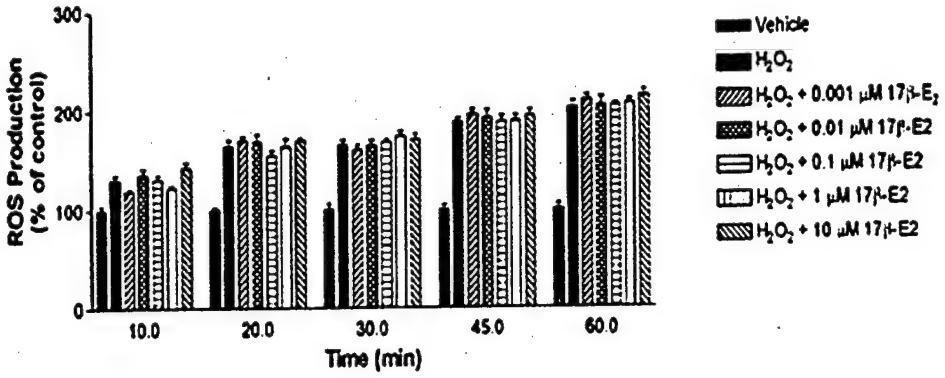

FIGURE 2. Effect of 2 hours of 17β-E_2 pretreatment on ROS production in HLE-B3 cells after 100 μM H_2O_2 treatment. ROS production was measured at indicated time points. Data are expressed as a percentage of vehicle control levels and represent mean ± SEM (n = 8).

ble $\Delta\Psi_m$ collapse when the estradiols were present, or conversely, under comparable Ca^{2+} loading, a larger portion of the mitochondrial population retained its membrane potential in the presence of estradiols. Note also that the magnitude of the $\Delta\Psi_m$ collapse in the presence of 17β-E_2, expressed as total AUC, was lower than with buffer and 17α-E_2, even at the highest ionomycin concentrations (Fig. 5).

Similar responses were observed when HLECs were exposed to 2.5 mM H_2O_2, as an acute cytotoxic stimulus (Fig. 6). As might be expected, the amount of H_2O_2 needed to collapse $\Delta\Psi_m$ acutely (response within seconds) was substantially more than the concentration necessary in the long-term cytotoxicity studies. In any event, the magnitude of $\Delta\Psi_m$ collapse induced by H_2O_2 was moderated by both 17β- and 17α-E_2 at 0.5 μM (preincubation for 30 minutes), although this occurred predominantly at higher H_2O_2 concentrations (0.1 and 1 mM). For example, EC_{50} was not significantly different between control and estradiol treatments, but one-way ANOVA of the total AUC during the response reveals significant moderation of $\Delta\Psi_m$ collapse by both 17β- and 17α-E_2 ($F = 3.9$, $P < 0.03$).

To model less acute oxidative stress that is likely to be more physiologically relevant, HLECs were incubated for 6 hours with 10 μM H_2O_2, with or without 17β- or 17α-E2, and then challenged with a Ca^{2+} load through application of ionomycin described earlier. 17β- or 17α-E_2 moderated $\Delta\Psi_m$ collapse, repressing the magnitude of the response more than its rate (Figs. 7, 8). In all three replicates, the estradiols consistently increase the EC_{50} for ionomycin (Fig. 8; Table 1). To control for potential effects of the compounds on cell growth or loss during the prolonged (6-hour) preincubation, the AUC data from each well were normalized to the cell density in that well at the start of the observations by dividing the AUC by the magnitude of quenching of the initial fluorescence signal by the potentiometric dye (A, initial signal; B, signal after quenching). Thus, AUC/($A - B$) provides an index of compound efficiency that is independent of effects on cell viability.[34]

Evidence that 17β-E_2 Protection against Oxidative Stress Is Not Mediated by Binding to Estrogen Receptors

To exclude a possible connection for ER-dependent binding with the protection afforded by 17β-E_2 against H_2O_2 induced cytotoxicity, two nonfeminizing estrogens that exhibit marginal (17α-E_2) or no (Ent-E_2) binding capacity to ERs were examined. Compared with the potent natural estrogen 17β-E_2, 17α-E_2 binds weakly to ERs, and the 17α-E_2–ER complex only transiently binds to the estrogen-responsive element.[28,35–37] Ent-17β-E_2, the enantiomer of 17β-E_2, has identical physiochemical properties as 17β-E_2, with the crucial exception that it is incapable of interacting with other stereospecific molecules, such as the ERs.[28] At 100 nM, 17β-E_2 improved cell survival from 32% ± 3% to 76% ± 3%. Both 17α-E_2 and Ent-E_2 displayed equivalent effectiveness against H_2O_2-induced cytotoxicity. 17α-E_2 increased cell survival to 73% ± 3% and Ent-E_2 enhanced cell viability to 79% ± 4% (Fig. 9), suggesting that the protective action of 17β-E_2 is not mediated by classic ERs.

To further exclude a role for ERs in estrogen protection against H_2O_2-induced cytotoxicity, an ER antagonist ICI 182,780 was examined because of its ability to prevent 17β-E_2 from binding to ERs. The structure of ICI 182,780 is similar to 17β-E_2, and it effectively competes with 17β-E_2 for binding to ERs. As shown in Figure 10, 100 μM H_2O_2 killed 79% ± 8% of cells, whereas 100 nM 17β-E_2 protected 77% ± 5% of cells from the H_2O_2 insult. Coadministration of ICI 182,780 and 17β-E_2 did not block the protection provided by17β-E_2 against H_2O_2 insult. At the same time, administration of 100 nM ICI 182,780 alone increased cell survival to 65% ± 6%, a result not entirely unexpected, because ICI 182,780 is structurally similar to 17β-E_2. Taken together, these data support our contention that the protection against H_2O_2 toxicity afforded by17β-E_2 is not mediated by ERs.

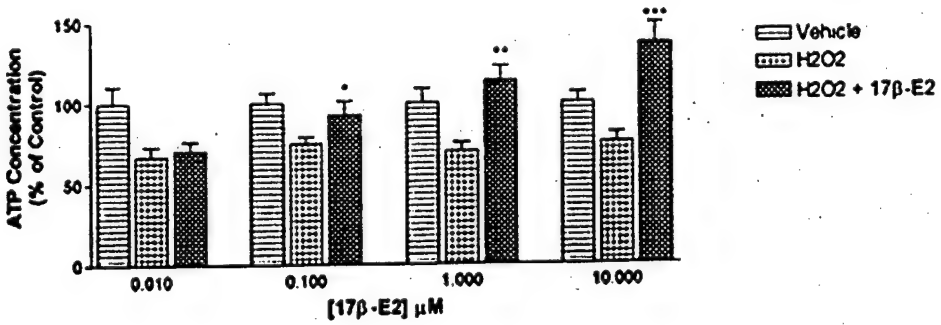

FIGURE 3. Effect of 2 hours of pretreatment with 17β-E_2 on ATP levels in HLE-B3 cells treated for 90 minutes with 100 μM H_2O_2. The vehicle group was pretreated with the equal amount of vehicle. Data are expressed as a percentage of normal levels and represent the mean ± SEM of determinations made in six to eight cultures per group. *$P < 0.05$ versus respective vehicle group; **$P < 0.05$ versus respective H_2O_2 group; ***$P < 0.05$ versus respective H_2O_2 group.

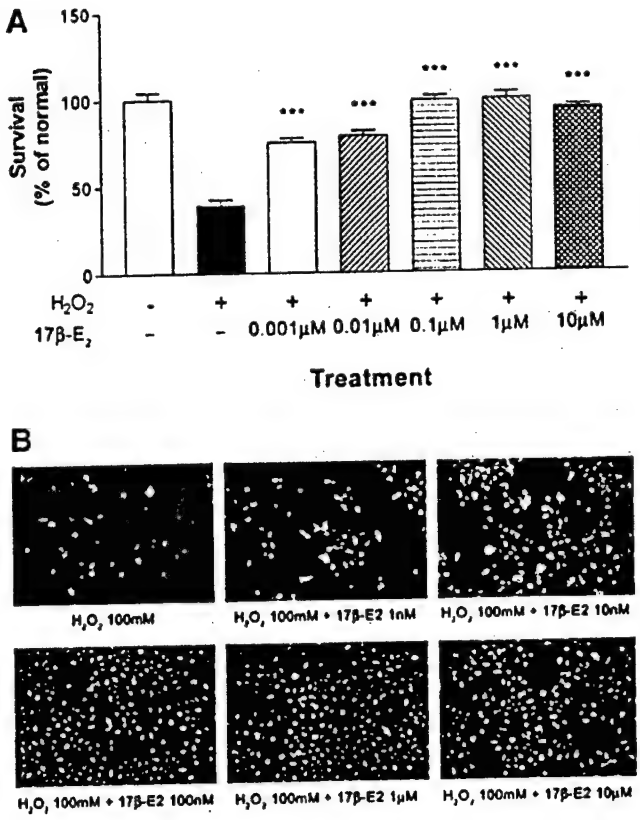

FIGURE 4. (a) Dose-dependent effects of 2 hours of pretreatment with 17β-E_2 on viability of HLE-B3 cells at 8 hours after 100 μM H_2O_2 treatment. The vehicle group (−H_2O_2 and 17β-E_2) was pretreated with the equal amount of vehicle. Data are expressed as a percentage of cells surviving and represent the mean ± SEM (n = 8). ***$P < 0.001$ versus the H_2O_2 group. (b) Photomicrographs of the dose-dependent effects of 2 hours of pretreatment with 17β-E_2 in HLE-B3 cells at 8 hours of 100 μM H_2O_2 treatment in calcein AM-loaded cells.

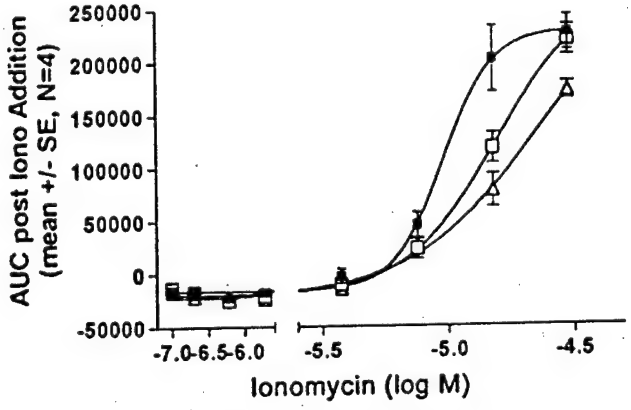

FIGURE 5. Effect of 17β-E_2 (△) and 17α-E_2 (□) on the collapse of $\Delta\Psi_m$, resulting from ionomycin-induced calcium loading. Both estradiols (0.5 μM, 5 minutes of preincubation) significantly moderated the magnitude of $\Delta\Psi$m collapse compared with the control (■), as reflected by the EC_{50} of 2.31 and 1.55 μM for 17β-E_2 and 17α-E_2, respectively, compared with control EC_{50} of 0.95 μM. Data are expressed as the AUC after addition of ionomycin and represent the mean ± SEM of four wells per group. When SEM bars are not shown, they are obscured by the symbol.

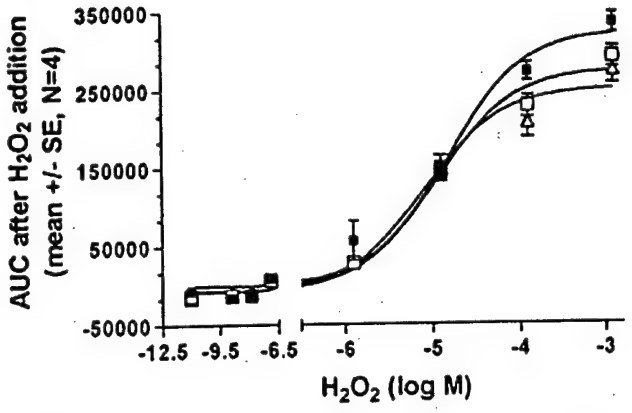

FIGURE 6. Effect of 17β-E_2 (△) and 17α-E_2 (□) on the acute collapse of $\Delta\Psi_m$, resulting from H_2O_2 exposure. Both estradiols (0.5 μM with 30 minutes of preincubation) significantly moderated the magnitude of $\Delta\Psi_m$ collapse compared with the control (■), as reflected by significant differences in the AUCs. In this case, there were no significant differences between the EC_{50} values (0.8, 1.1, and 1.5 μM for 17β-E_2, 17α-E_2, and control, respectively), although one-way ANOVA of the AUCs reveals that both estradiols significantly suppressed the $\Delta\Psi_m$ collapse caused by H_2O_2 ($F = 3.9$, $P < 0.03$). This suggests that the differences between the treatments are dominated by divergence at the highest H_2O_2 concentrations. Regression coefficients (r^2) are 0.96, 0.97, and 0.96 for 17β-E_2, 17α-E_2, and control, respectively. Data are expressed as the AUC after addition of H_2O_2 and represent the mean ± SEM of four wells per group. When SEM bars are not shown, they are obscured by the symbol.

DISCUSSION

We report, for the first time, that the cell death induced in HLECs by H_2O_2 is associated with production of intracellular ROS, collapse of $\Delta\Psi_m$, and profound depletion of ATP and that estrogens potently protected against collapse of $\Delta\Psi_m$, ATP

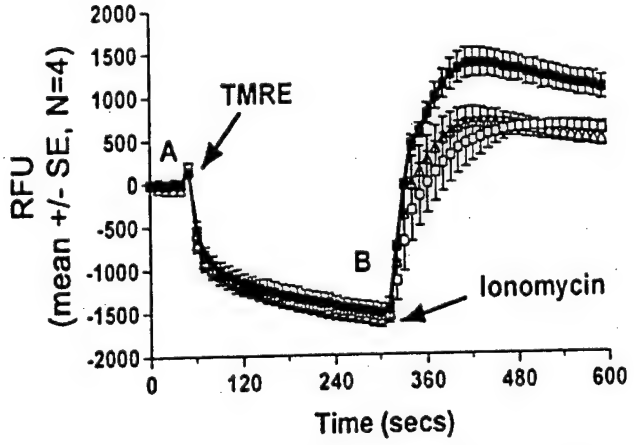

FIGURE 7. Time course of the effects of 17β-E_2 (△), 17α-E_2 (□) or control buffer (no estrogen, ■) on ionomycin-mediated $\Delta\Psi_m$ collapse in H_2O_2-treated cells, using the FRET assay. To more closely mimic the in vivo situation, before Ca^{2+} challenge, the lens cells were incubated for 6 hours with 0.5 μM estradiols plus 10 μM H_2O_2. Addition of a potentiometric dye, TMRE (*arrow* at 50 seconds) quenched the initial NAO signal (A), whereas addition of ionomycin (5 μM, *arrow* at 320 seconds) collapsed $\Delta\Psi_m$, thereby releasing the dye from the mitochondria. Efflux of the dye dequenched the NAO, as is seen by the sharp recovery of the NAO signal.[32,33] Data are expressed as mean RFU. Treatments were compared using the AUCs after Ca^{2+} (or H_2O_2) challenge.

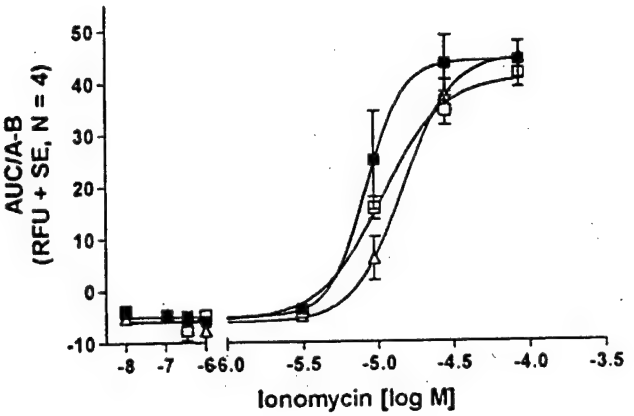

FIGURE 8. Effects of estradiol on $\Delta\Psi_m$ collapse after long-term exposure to H_2O_2. Before Ca^{2+} challenge, the lens cells were treated as in Figure 7. In this protocol, both 17β-E_2 (△) and 17α-E_2 (□) moderated the acute collapse of $\Delta\Psi_m$ resulting from exposure to ionomycin, compared with the control (■). This was reflected by a consistent increase in EC_{50} over the control (Table 1), indicating mitochondrial stabilization by estradiols. Regression coefficient values are in Table 1. Data are expressed as AUC/$A - B$ after addition of ionomycin and represent the mean ± SEM of four wells per group. When SEM bars are not shown, they are obscured by the symbol.

depletion, and cell death without affecting production of ROS such as H_2O_2, $RO_2^{\cdot}$, $RO^{\cdot}$, $OH^{\cdot}$, HOCl, and $ONOO^-$. Collectively, these data suggest that the reported protection from cataracts afforded by HRT in postmenopausal women[7-12] is due to these cytoprotective effects against H_2O_2 toxicities in lens epithelial cells.

H_2O_2 is a potent diffusible pro-oxidant that initiates a series of oxidative events in cells.[38,39] We observed that HLECs adaptively responded to a low concentration (50 μM) of H_2O_2, as evidenced by a modest increase in ROS, maintenance of stable, albeit lower, concentrations of ATP, and relative resistance to cell death. In contrast, the higher (100 μM) concentration of H_2O_2 was associated with a delayed but profound increase in ROS, a rapid and marked decline in ATP concentrations, and nearly complete cell death within 8 to 12 hours. This higher concentration of H_2O_2 is clearly a pathologic insult, from which cells failed to recover under the conditions of this study.

Our first assessment of the effects of 17β-E_2 was on production of ROS, using an indicator that detected soluble ROSs. In this assay, 17β-E_2 at concentrations ranging from low physiological (1 nM) to pharmacologic (10 μM) were ineffective in changing the ROS response to H_2O_2. Clearly, the cytoprotective effects of 17β-E_2 in this cell line are not dependent on its ability to affect the production or clearance of soluble ROS. A

TABLE 1. EC_{50} and Regression Correlation Coefficient for Effect of Estrogens in Long-Term Exposure to H_2O_2

Exp.	Buffer	17α-E_2	17β-E_2
1	0.8 (0.89)	1.1 (0.96)	1.4 (0.97)
2	2.3 (0.93)	2.8 (0.96)	3.0 (0.94)
3	2.1 (0.92)	3.1 (0.85)	3.7 (0.84)

EC_{50} values (in micromolar) and correlation coefficients in brackets from three repetitions of the prolonged-treatment experiment in Figure 8. Because these values are a function of cell density, dye intensity, and optical aberrations (among other variables), they are expected to vary between experiments. Note, however, that despite variability, in all iterations the estradiols (0.5 μM) increased the amount of Ca^{2+} necessary to induce $\Delta\psi_m$ collapse (i.e., improve mitochondrial stability).

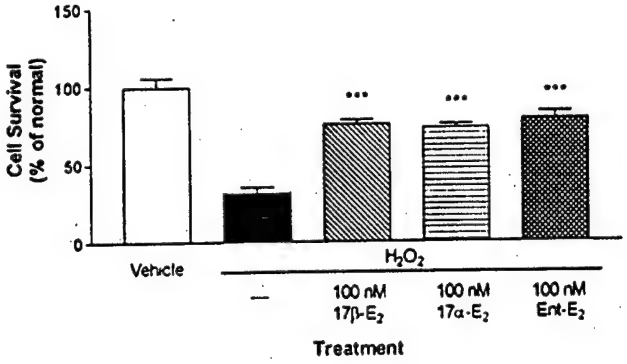

FIGURE 9. Effects of 2 hours of pretreatment with 17β-E_2, 17α-E_2, and Ent-E_2 on cell viability in HLE-B3 cells for 8 hours at 100 μM H_2O_2 treatment. Data are expressed as a percentage of the vehicle group and represent the mean ± SEM (n = 8). ***$P < 0.001$ versus the H_2O_2 group.

possibility that we have not explored in the present study is that estrogens may affect lipid peroxidation, without affecting soluble ROS. Estrogens are lipid soluble and preferentially penetrate in cellular membranes.[40] In neuronal cultures, physiological concentrations of 17β-E_2 effectively reduce lipid peroxidation.[22,41] However, it is clear from our data that in HLECs, soluble ROS are not influenced by exposure to estrogen.

The ATP depletion induced by H_2O_2 treatment no doubt reflects at least two actions of the pro-oxidant: interruption of oxidative production of ATP with the concomitant depletion of the energy-containing molecule as a result of attempts to repair damage caused by H_2O_2. Either or both mechanisms may be involved in the depletion of ATP, although we have observed that H_2O_2 causes a profound downregulation in several oxidative phosphorylation enzyme transcripts (Cammarata PR, Moor AN, unpublished observations, 2002), an effect that would certainly help undermine ATP production. Moreover, we do not exclude the well-known dramatic inactivation of glyceraldehyde-3-phosphate dehydrogenase by H_2O_2 as part of the explanation for the decline in intracellular ATP.[42] Whether estradiol prevents the inactivation of glyceraldehyde-3-phosphate dehydrogenase by peroxide, thereby contributing to the restoration of intracellular ATP, is currently under investigation.

On shorter time scales, it is well known that oxidative insult readily represses electron transport efficiency and oxidative

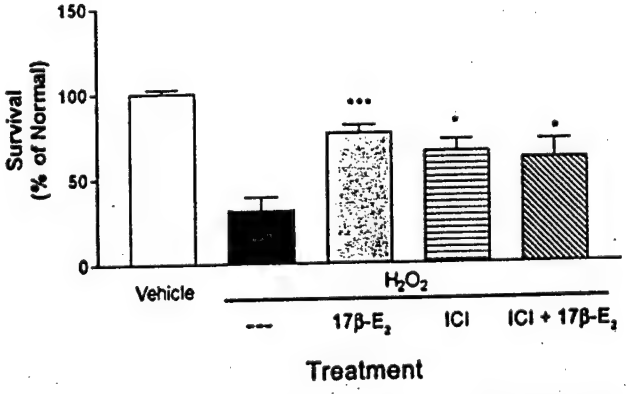

FIGURE 10. Effects of ICI 182,780 on 17β-E_2 protection in HLE-B3 cells after 8 hours of 100 μM H_2O_2 treatment. Data are expressed as a percentage of cells surviving compared with the vehicle group and represent the mean ± SEM (n = 7-8). *$P < 0.05$ and ***$P < 0.001$ versus the H_2O_2 group.

phosphorylation, primarily by inactivating both the Fe-S reaction centers of several of the electron transport respiratory centers, and heme moieties in the cytochromes.[39,43] In addition, even without the downregulation of mitochondrial gene expression noted earlier, the data show that H_2O_2 directly collapses $\Delta\Psi_m$ in HLECs, an event that not only eliminates the driving force for mitochondrial ATP production, but that also exacerbates subsequent free radical production.[39,43]

Damage to mitochondria can lead to deficiency in ATP production and to a concomitant increase in production of ROS that can overwhelm cellular antioxidant defense systems. Under conditions of oxidative stress, mitochondria undergo a catastrophic, irreversible loss of the impermeability of the inner mitochondrial membrane that causes a complete collapse of $\Delta\Psi_m$, a process called permeability transition (PT).[44] Accelerated mitochondrial radical production compromises cellular and mitochondrial integrity by inducing peroxidation of membrane lipids and impeding oxidative phosphorylation. The resultant acute loss of ATP causes the transmembrane ion-dependent ATPases to fail, thereby precipitating cell death from osmotic failure.[39,45]

17β-E_2 was effective in protecting cellular ATP levels and in protecting HLECs from death. A dose-dependent increase in cellular ATP levels was observed from 100 nM to 10 μM. These data suggest that at pharmacologic concentrations of estrogens, cellular ATP is preserved. Inasmuch as ATP is essential for normal cellular function, including its survival, this action of 17β-E_2 may be necessary, but insufficient for the observed cytoprotective effects. Indeed, it appears that the cytoprotective effects of 17β-E_2 occur at concentrations lower than those needed for ATP maintenance. As such, other actions of 17β-E_2 are involved in its cytoprotective effects. Although we do not know the precise mechanism of the cytoprotective effects of estrogens in HLECs, in neurons a plethora of cellular responses to the steroid have been reported, including the protection of mitochondrial function, stimulation of antiapoptotic proteins, and stimulation of protective signaling pathways.[46-51]

The data in the current study indicate that both 17α- and β-E_2 equipotently increased the amount of Ca^{2+} or H_2O_2 necessary to collapse $\Delta\Psi_m$ in HLECs, effectively stabilizing mitochondrial integrity and preserving function under pathogenic conditions. This effect does not require prolonged exposure to the estradiols—it became apparent in 5- and 30-minute incubations—yet it was also apparent after a 6-hour preincubation. The result is that, at a given Ca^{2+} or oxidative load, a larger portion of the mitochondrial population retains $\Delta\Psi_m$ and hence continues to function. Such a response readily explains preservation of ATP levels by estradiols during exposure to H_2O_2, as well as repression of cell death through both necrosis and apoptosis under these conditions.

The mitoprotective effects of the estradiols shown in the $\Delta\Psi_m$ assay could be due to any combination of the mechanisms of action known for this class of compounds[46-51] such as membrane stabilization,[40] which is particularly germane to the retention of $\Delta\Psi_m$. Indeed, the moderation by estrogens of $\Delta\Psi_m$ collapse could be due to a repression of Ca^{2+} uptake into the mitochondria through the uniporter, to increased Ca^{2+} efflux from the mitochondria, or to a direct membrane-stabilization effect, all of which would yield similar-appearing responses in this assay. In addition, although both 17α- and β-E_2 equipotently repressed the magnitude of $\Delta\Psi_m$ collapse induced by either Ca^{2+} or H_2O_2 in the FRET assay (Figs. 5, 6, 7, 8), the data do not permit distinguishing a modest effect in most of the mitochondrial population from a profound effect in a smaller mitochondrial subpopulation. It is apparent, however, that in the absence of additional stressors, the estrogens alone do not dissipate or hyperpolarize $\Delta\Psi_m$, as is reflected by comparable amounts of initial quenching, seen as coincidence of the curves between 100 and 300 seconds before the addition of ionomycin (Fig. 7). At the very least, this suggests that the estrogens do not exert their protective effects by uncoupling electron transport, a mechanism known to protect neuronal cells from oxidative stress and Ca^{2+} loading associated with glutamate excitotoxicity.[52]

Our results argue against a primary involvement of ERs in the observed effects of estrogens. However, we have, in fact, confirmed the positive presence of ER-α and -β in the cultured HLEC strain HLE-B3 by RT-PCR analysis and subsequent verification of the PCR products by sequence analysis and Southern blot with specific internal oligonucleotides to the directed primer pairs, as well as by immunofluorescence techniques (manuscript in preparation). Three estrogens, 17β-, 17α-, and Ent-E_2, that differ by as much as 32-fold in their affinity for either ER-α or -β,[28,35-37] have equivalent effects on HLEC survival and the action of 17β-E_2 was not antagonized by the prototypic ER antagonist ICI 182,780. The ICI compound itself exerted cytoprotective activity, probably the result of its phenolic A ring, a requirement for cytoprotection by estrogens.[21,23,45] In this respect, a recent study by Han et al.,[53] demonstrated that the protective potency of various estrogens was dependent on the precise estrogenic structure. Whereas 17α-E_2, a phenolic ring estrogen, acted similar to the antioxidants taurine and vitamin C against the peroxide-induced damage to cultured rabbit renal proximal tubule cells, 17β-E_2, a catecholic estrogen, behaved in a manner similar to the iron chelators deferoxamine and phenanthroline. In this regard, it is important to further point out that superoxide dismutase mimics, in particular, TEMPOL (Sigma),[54] prevents Fe^{+2}-mediated generation of the damaging hydroxyl radical, by reacting with superoxide, thus preventing recycling of Fe^{+3} to Fe^{+2}, while deferoxamine chelates intracellular Fe^{+3} and prevents its reduction to Fe^{+2}. We cannot at this time rule out the possibility that 17β-E_2, like the superoxide dismutase (SOD) mimic TEMPOL or deferoxamine, acts by limiting the availability of Fe^{+2} and thereby prevents certain damaging effects of H_2O_2. Irrespective of the precise mode of action of 17β-E_2, the study by Han et al.,[53] raises the interesting possibility that various estrogens have differential cytoprotective potential, and by inference, disparity in their mechanisms of action. Of immediate relevance to our studies, however, Han et al., like us, conclude that "these cytoprotective effects of estrogens are not dependent on classical estrogen receptors."[53]

In contrast, a recent study by Davis et al.,[55] argues that estrogen protection in the eye may result from direct interactions with its ocular ERs. Studies in their transgenic mouse model, which express ER-Δ, a dominant-negative form of ER-α that inhibits ER-α function, show spontaneous cortical cataracts that progress with age in transgene-positive women after puberty.

Collectively, our studies demonstrate that estrogens are potent cytoprotectants that preserve mitochondrial function during oxidant insult in HLECs in culture. These results indicate that estrogens may be useful therapies for the prevention of cataracts in postmenopausal women and that nonfeminizing estrogens could provide similar protection in men.

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Appendix K

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Testosterone increases neurotoxicity of glutamate in vitro and ischemia-reperfusion injury in an animal model

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Yang, Shao-Hua, Evelyn Perez, Jason Cutright, Ran Liu, Zhen He, Arthur L. Day, and James W. Simpkins. Testosterone increases neurotoxicity of glutamate in vitro and ischemia-reperfusion injury in an animal model. *J Appl Physiol* 92: 195–201, 2002.—Increasing evidence has demonstrated striking sex differences in the outcome of neurological injury. Whereas estrogens contribute to these differences by attenuating neurotoxicity and ischemia-reperfusion injury, the effects of testosterone are unclear. The present study was undertaken to determine the effects of testosterone on neuronal injury in both a cell-culture model and a rodent ischemia-reperfusion model. Glutamate-induced HT-22 cell-death model was used to evaluate the effects of testosterone on cell survival. Testosterone was shown to significantly increase the toxicity of glutamate at a 10 μM concentration, whereas 17β-estradiol significantly attenuated the toxicity at the same concentration. In a rodent stroke model, ischemia-reperfusion injury was induced by temporal middle cerebral artery occlusion (MCAO) for 1 h and reperfusion for 24 h. To avoid the stress-related testosterone reduction, male rats were castrated and testosterone was replaced by testosterone pellet implantation. Testosterone pellets were removed at 1, 2, 4, or 6 h before MCAO to determine the duration of acute testosterone depletion effects on infarct volume. Ischemic lesion volume was significantly decreased from 239.6 ± 25.9 mm^3 in control to 122.5 ± 28.6 mm^3 when testosterone pellets were removed at 6 h before MCAO. Reduction of lesion volume was associated with amelioration of the hyperemia during reperfusion. Our in vitro and in vivo studies suggest that sex differences in response to brain injury are partly due to the consequence of damaging effects of testosterone.

androgen; stroke; neuroprotection

GONADAL STEROID HORMONES such as androgens and estrogens may affect various target tissues throughout the body, including central nervous system. Clinical evidence has demonstrated striking sex differences in the incidence and outcome of stroke (27), which precipitated the studies of the potential impact of gonadal steroid hormones in disturbances of the central nervous system. A major focus in basic and clinical research in the last decade has been related to the activities of estrogens. Although the impact of postmenopausal estrogen-replacement therapy on stroke prevention and stroke severity remains inconsistent (7, 26), data from experimental studies in laboratory animals suggest that estrogens may have neuroprotective properties (3, 12, 33, 42), which have led to a growing appreciation of the positive impact of estrogens on the central nervous system. In contrast, effects of androgens on the central nervous system are much less studied.

Testosterone has been shown to be a survival factor for axotomized motoneurons and promotes motor axon regeneration (21, 22). Recently, several in vitro studies suggested that testosterone possessed neuroprotective effects on cerebellar granule neuron (1, 2). In view of the proposed neuroprotective effects both of estrogens and androgens, effects of sex difference on the outcome of stroke (3, 27, 44) could not be explained by sex hormones. We have previously reported that chronic testosterone replacement increased while chronic castration and chronic 17β-estradiol treatment decreased ischemic damage related to middle cerebral artery occlusion (MCAO) in male rats (19) and that the decrease of ischemic lesion volume with chronic 17β-estradiol treatment was associated with a marked reduction of testosterone level in intact males (19). In the present study, effects of acute testosterone depletion on ischemic stroke were evaluated. Our objective was twofold. First, direct effects of testosterone on neuronal survival were evaluated in a HT-22 cell-culture model using glutamate insult. Second, effects of acute testosterone depletion on ischemic lesion volume from MCAO were assessed in male rats. Our strategy was to compare the ischemic lesion volume from MCAO between testosterone depletion animals and animals with physiological level testosterone. Sustained physiological testosterone levels were obtained by castration and steroid pellet replacement technique, which our

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laboratory has previously reported (19). Acute testosterone depletion was achieved by pellets withdrawn 2 days after castration and testosterone pellet implantation, whereas sham withdrawal was used to maintain physiological testosterone levels in control. By using this strategy, the effects of timed depletion of testosterone before ischemic insult on the lesion volume and regional cerebral blood flow (CBF) from temporary MCAO were assessed in male rats.

MATERIALS AND METHODS

Cell culture and treatment. HT-22 cells (gift from David Schubert, Salk Institute, San Diego, CA), which are a murine hippocampal cell line, were maintained in DMEM media (GIBCO, Gaithersburg, PA) supplemented with 10% charcoal-stripped fetal bovine serum (HyClone, Logan, UT) and 20 μg/ml gentamycin under standard cell culture conditions (5% CO_2, 95% air, 37°C). HT-22 cells (*passages 18–25*) were seeded into Nunc 96 well plates at a density of 5,000 cells/well.

Testosterone and 17β-estradiol were initially dissolved in absolute ethanol and diluted in DMEM media to the final concentration of 0.01–10 μM. Exposure to testosterone and 17β-estradiol was initiated immediately before the addition of glutamate. Ethanol was used at a final concentration of 0.1% as vehicle control. Glutamate was diluted to a final concentration of 10 mM in culture media, and cells were exposed to glutamate for ~24 h. All cell culture experiments are repeated at least three times.

Cell viability assay. Cells were exposed to steroids and glutamate for ~24 h, and then cell viability was determined by calcein AM (Molecular Probes, Eugene, OR), an assay that measures cellular esterase activity and plasma membrane integrity. Wells were rinsed with PBS, after which a 25 μM solution of calcein AM in PBS was added. After incubation at room temperature for 15 min, fluorescence was determined (excitation = 485, emission = 530). Raw data were obtained as relative fluorescence units. All data were then normalized to percentage of cells killed, as calculated by treatment value/control value × 100.

Experimental animals. Male Charles River Sprague-Dawley rats (250 g, Wilmington, MA) were maintained in laboratory acclimatization for 3 days before surgery. All animal procedures were approved by the University of North Texas Health Science Center Animal Care and Use Committee and University of Florida Animal Care and Use Committee.

Testosterone concentration in testosterone-replacement and withdrawal animals. To determine the effect of testosterone pellet implantation on serum testosterone concentration and the time course of testosterone reduction after pellet withdrawal, bilateral castration was performed under methoxyflurane inhalant anesthesia, and two 15-mm-long testosterone Silastic pellets containing crystalline steroid were implanted subcutaneously immediately thereafter. Blood samples (0.5 ml each time) were taken via jugular vein at 24 (n = 4) and 48 h (n = 4) after the implantation of steroid pellets under methoxyflurane inhalant anesthesia. Then the pellets were removed and blood samples were taken via jugular vein at 1, 2, 4, 8, 12, and 24 h (n = 4 each time point) after steroid pellet removal. Serum was separated from blood cells by centrifugation and stored frozen (−20°C). Serum testosterone concentrations were determined by using duplicate serum aliquots in a radioimmunoassay (Diagnostic Systems Laboratories, Los Angeles, CA). Animals used for testosterone assessment were not used for ischemia outcome studies.

Experiment protocol. Two days after castration and testosterone pellet implantation, ischemic stroke was induced in animals after testosterone pellet removal or sham removal. Pellets were removed in the testosterone depletion groups at 1 (n = 7), 2 (n = 5), 4 (n = 5), or 6 h (n = 5) before ischemia under methoxyflurane inhalant anesthesia. Sham pellet removal was performed in the physiological testosterone level group as control in the same condition as pellet removal at 1 (n = 5), 4 (n = 5), and 6 h (n = 5) before MCAO. Ischemic stroke was induced by MCAO described as before (18, 31). Briefly, animals were anesthetized by intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). Rectal temperature was monitored and maintained between 36.5 and 37.5°C during the procedure. With the aid of an operating microscope, the left common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed through a midline cervical skin incision. CCA and ECA were permanently cauterized. A 3–0 monofilament suture was introduced into the ICA via ECA lumen and advanced until resistance was encountered. The distance between the CCA bifurcation and the resistive point was ~1.9 cm. The middle cerebral artery was occluded for 1 h, and then the suture was withdrawn for reperfusion. ICA was coagulated, and the skin incision was closed.

Animals in each group were decapitated 24 h after reperfusion. Then the brain was harvested and placed in a metallic brain matrix for tissue slicing (Harvard Apparatus, Holliston, MA). Seven slices were made at 3, 5, 7, 9, 11, 13, and 15 mm posterior to the olfactory bulb. Each slice was incubated for 30 min in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) in physiological saline at 37°C and then fixed in 10% formalin. Stained slices were photographed by a digital camera (Sony MVC-FD5, Tokyo, Japan) and subsequently measured for the surface area of the slices and the ischemic lesion (Image-Pro Plus 4.1, Media Cybernetics, Silver Spring, MD).

Regional CBF measurement and physiological parameters monitor. In a separate study, MCAO was induced 6 h after pellet (n = 6) or sham removal (n = 6). Left femoral artery was canalized and connected to a blood pressure monitor. Arterial blood samples (150 μl each time) were taken before, 30 min during, and 30 min after MCAO, respectively. Physiological parameters were measured by an ISTAT portable clinical analyzer (East Windsor, NJ).

Hydrogen clearance blood flowmeter (Digital UH meters, Unique Medical, Tokyo, Japan) was used for regional CBF measurement. Two Teflon-coated platinum probes were stereotaxically inserted into the core area of ischemia (0.5 mm posterior bregma, 4 mm lateral, and 5 mm depth). Regional CBF was monitored bilaterally during occlusion and within 30 min after reperfusion in testosterone pellet removal and sham removal groups.

Statistical analysis. All data are presented as means ± SE. Cell death, CBF, ischemic volumes, and physiological parameters in each group were compared by one-way ANOVA followed by Tukey tests. A probability of <0.05 was considered significant.

RESULTS

Effect of testosterone and 17β-estradiol on glutamate toxicity. Ten micromolar testosterone significantly increased glutamate toxicity to 87.5 ± 3.7% of cells killed, compared with 71.9 ± 6.9% at 0 μM testosterone. Opposite to the deleterious effect of testosterone,

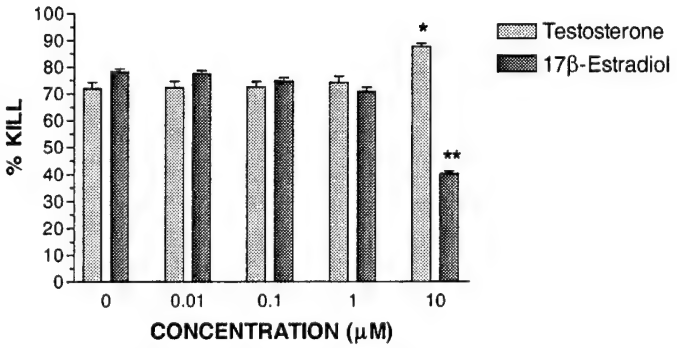

Fig. 1. Effect of testosterone and 17β-estradiol on glutamate toxicity. HT-22 cells were exposed to 10 mM glutamate for ~24 h. Cell viability was determined by calcein assay. All data were normalized of percentage of cells killed (%kill) as calculated by treatment value/control value × 100. Values are means ± SE. *$P < 0.05$ vs. 0 μM testosterone. **$P < 0.05$ vs. 0 μM 17β-estradiol.

10 μM 17β-estradiol ameliorated glutamate toxicity to 40.3 ± 3.1% of cells killed, compared with 78.3 ± 3.3% at 0 μM 17β-estradiol (Fig. 1).

Testosterone concentrations in testosterone replacement and withdrawal animals. Subcutaneous implantation of testosterone pellets increased serum testosterone concentrations to 2.58 ± 0.47 and 1.83 ± 0.13 ng/ml at 1 and 2 days after implantation, respectively, both of which are within the reported physiological range of testosterone in male rats (Fig. 2). Serum testosterone concentrations decreased to 0.24 ± 0.01 ng/ml at 1 h after removal of the pellets. Thereafter, testosterone concentrations decreased to <0.08 ng/ml, the limits of sensitivity of the assay (Fig. 2).

Effect of testosterone on ischemic lesion volume. Ischemic lesion volume was significantly decreased when testosterone pellets were removed at 6 h before MCAO. Lesion volume was 217.8 ± 24.69, 192.6 ± 13.90, 151.3 ± 45.54, and 122.5 ± 28.62 mm^3 at 1, 2, 4, and 6 h after pellet removal, respectively, compared with 239.6 ± 25.89 mm^3 in control animals in which physiological testosterone concentrations were maintained (Fig. 3). As no differences in ischemic lesion volume were found between sham pellet removal animals at 1, 4, or 6 h before MCAO, all sham pellet removal animals were pooled together as controls.

Effect of testosterone on blood pressure, gases, pH, ions, and regional CBF. Physiological parameters are shown in Table 1. There were no significant differences between testosterone and testosterone-depletion groups for any parameters measured.

Regional CBF decreased to 8.7 ± 2.1 and 7.5 ± 1.9 $ml \cdot min^{-1} \cdot 100\ g\ tissue^{-1}$ during MCAO in the testosterone and testosterone-depletion groups, respectively. Hyperemia was observed during reperfusion in the testosterone group, which showed a CBF of 82.2 ± 12.2 $ml \cdot min^{-1} \cdot 100\ g\ tissue^{-1}$ compared with the nonischemic side in the testosterone and testosterone-depletion groups ($P < 0.05$), which had CBF of 32.0 ± 1.7 and 46.0 ± 3.6 $ml \cdot min^{-1} \cdot 100\ g\ tissue^{-1}$, respectively. In the testosterone-depletion group, no significant hyperemia was observed (Fig. 4).

DISCUSSION

Brain injury by transient global brain ischemia (cardiac arrest) and focal brain ischemia (ischemia stroke) is the leading cause of serious and long-term disability in the US (40). Striking differences in the incidence and outcome of stroke between males and females have been suggested to have resulted from the neuroprotective effects of estrogens (3, 12, 26, 33, 42). In the present study, testosterone was shown to posses deleterious effects on ischemic stroke in a focal ischemia model, whereas acute testosterone depletion exerts neuroprotective effects, which suggests that effects of testosterone could also contribute to these gender differences of stroke.

Experimental focal brain ischemia is one of the models most widely used to test the neuroprotective effects of estrogens in vivo. Protective effects of estrogens have been documented by using MCAO model (3, 12, 33, 42). In male rats, castration has also been reported to decrease ischemia-reperfusion injury in this model (19), whereas another report showed that castration

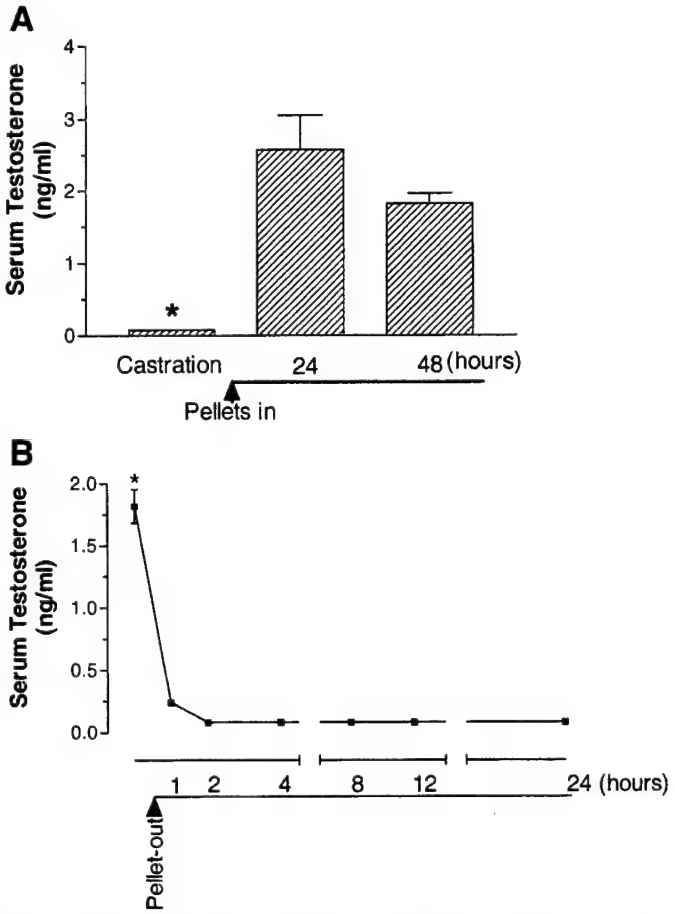

Fig. 2. Testosterone concentrations in testosterone-replacement and -depletion animals. *A*: rats ($n = 4$) were castrated, and two testosterone pellets were implanted subcutaneously. Testosterone concentrations were maintained at 2.58 ± 0.47 and 1.83 ± 0.13 ng/ml at 1 and 2 days after implantation, respectively. *B*: testosterone concentration decreased to 0.24 ± 0.01 ng/ml at 1 h after removal of the pellets. Thereafter, testosterone concentration decreased to <0.08 ng/ml. Values are means ± SE. *$P < 0.05$ vs. pellets in.

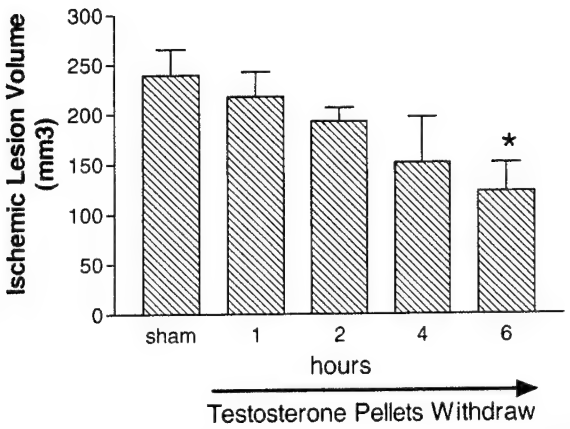

Fig. 3. Effect of testosterone depletion on ischemic lesion volume. Lesion volume was 217.8 ± 24.7, 192.6 ± 13.9, 151.3 ± 45.5, and 122.5 ± 28.6 mm^3 at 1 (n = 7), 2 (n = 5), 4 (n = 5), and 6 h (n = 5) after pellet removal, respectively, compared with 239.6 ± 25.9 mm^3 in control animals with testosterone (n = 15). All values are means ± SE. *P < 0.05 vs. sham.

did not affect the ischemia-reperfusion injury by using a similar model (36). Two reasons could attribute to the different result between these studies: *1*) difference in the duration of MCAO, which was 1 h in the former study compared with 2 h in the latter study; and *2*) wide range of testosterone concentrations in noncastrated animals in the latter study, which ranged from 0.05 to 1.62 ng/ml. The wide range of testosterone concentrations in the intact male animals could have resulted from the different kinds of stress and daily rhythms of testosterone. Testosterone had a daily rhythm in young male rats, with daily troughs as low as ~0.5 ng/ml and peaks as high as 2.0 ng/ml (32). In the present study, castration and testosterone-replacement techniques were used to evaluate the effects of acute testosterone depletion on ischemia-reperfusion injury. This technique produces a sustained physiologically relevant testosterone level and avoids the influence of stress and daily rhythms in testosterone levels. Testosterone levels decline rapidly in response to both physical and psychological stress (14), and testosterone levels are reduced in stroke patients (10, 16). Testosterone levels have been shown to be inversely associated with stroke severity and 6-mo mortality, whereas estradiol levels were not reduced in stroke patients (20). We have also shown that testosterone levels decrease significantly after MCAO (Fig. 5). Physiological consequences of this response are still unclear. It has been shown that adrenomedullary activation may be influenced by the stress-induced decline in testosterone (15). Testosterone receptor blockade using flutamide appeared to ameliorate the depressed adrenal function in males after trauma and severe hemorrhagic shock (5). Stress-induced testosterone reduction could positively influence stroke outcome through adrenomedullary activation. In the present study, acute depletion of testosterone significantly decreased the ischemic lesion volume, which suggests that stress-related testosterone reduction could be a protective response.

Interestingly, acute depletion of testosterone before ischemic insult caused a time-dependent improvement in MCAO outcome. One of the reasons for the time-dependent effects of testosterone depletion could have resulted from the delayed degradation of testosterone in the brain. Our previous study suggested that plasma testosterone was a primary determinant of the size of ischemic lesions following MCAO in male rats (19). The half-life of serum testosterone is very short, and serum testosterone decreased to an undetectable level within 2 h after pellet removal. Testosterone is highly hydrophobic and is cleared much more slowly from lipid-rich tissue, such as brain tissue, than from blood. So central nervous system effects of testosterone can persist after androgen depletion (13). Delayed effects of testosterone depletion also suggest that the effects could be mediated through a transcriptional mechanism, which could take 4 to 6 h to terminate.

Our data show that there was a similar CBF reduction in both the testosterone and testosterone-depletion groups during MCAO. Hyperemia was shown clearly in the ischemic side within 30 min after reperfusion compared with the contralateral side in the testosterone group but not in the testosterone-depletion group. This suggests that the deleterious effects of testosterone could be CBF related. Reactive hyperemia and delayed hyporemia have been found during reper-

Table 1. *Physiological parameters in rats subjected to transient MCAO*

	Testoterone			Testosterone Depletion		
	Before MCAO	During MCAO	After MCAO	Before MCAO	During MCAO	After MCAO
MABP, mmHg	87.0 ± 2.5	77.8 ± 2.5	87.0 ± 5.4	84.3 ± 1.9	82.0 ± 5.6	80.4 ± 2.7
P_{CO_2}, Torr	48.8 ± 2.9	46.9 ± 1.3	45.3 ± 1.3	56.1 ± 2.3	47.6 ± 4.8	42.1 ± 3.9
P_{O_2}, Torr	78.2 ± 7.2	73.0 ± 3.1	86.0 ± 4.3	67.2 ± 4.1	71.0 ± 7.2	80.6 ± 8.3
HCO_3, mmol/l	26.6 ± 0.4	25.8 ± 0.2	25.0 ± 0.3	27.0 ± 0.7	26.2 ± 1.1	25.2 ± 0.9
SO_2, %	93.2 ± 2.3	93.2 ± 0.9	95.7 ± 0.8	88.8 ± 2.9	90.8 ± 3.7	94.2 ± 1.8
pH	7.35 ± 0.02	7.35 ± 0.01	7.35 ± 0.01	7.29 ± 0.02	7.36 ± 0.03	7.39 ± 0.03
Hb, g/dl	14.4 ± 0.2	14.2 ± 0.5	14.6 ± 0.5	14.5 ± 0.2	14.0 ± 0.0	14.4 ± 0.2
Na^+, mmol/l	141.4 ± 0.9	140.0 ± 0.9	140.2 ± 1.6	141.2 ± 1.4	138.0 ± 1.1	139.8 ± 1.5
K^+, mmol/l	4.5 ± 0.1	5.3 ± 0.2	5.0 ± 0.3	4.8 ± 0.1	5.8 ± 0.2	5.1 ± 0.2

Values are means ± SE (n = 6 for each group). MCAO, middle cerebral artery occlusion; MABP, mean arterial blood pressure. There were no significant differences between treatment groups at any sampling time.

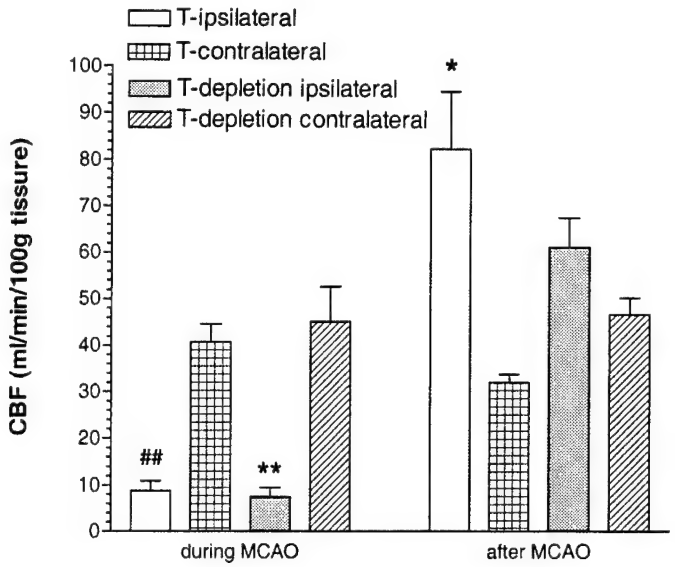

Fig. 4. Effect of testosterone on regional cerebral blood flow (CBF). T-ipsilateral, ischemic side in testosterone group; T-contralateral, contralateral side in testosterone group; T-depletion ipsilateral, ipsilateral side in testosterone-depletion group; T-depletion contralateral, contralateral side in testosterone-depletion group; MCAO, middle cerebral artery occlusion. Values are means ± SE. $^{*}P < 0.05$ vs. T-contralateral and T-depletion contralateral. $^{**}P < 0.01$ vs. T-depletion contralateral. $^{\#\#}P < 0.01$ vs. T-contralateral during MCAO.

fusion, and both are thought to be harmful to ischemic tissue (34, 37). Ischemic edema and blood-brain barrier disruption have been found to be exacerbated after acute reperfusion, which is related to the sudden surge reperfusion with hyperemia (23, 41). Gradual blood flow restoration could significantly reduce the exacerbation of ischemic edema and blood-brain barrier opening (17). As such, the damaging effects of testosterone could have partially resulted from reactive hyperemia during reperfusion.

The mechanism of testosterone's effect on CBF is unclear. Testosterone has been shown to be vasoactive in the peripheral artery system. Treatment with testosterone causes a vasorelaxant response in rabbit coronary arteries (43). Other studies (29, 39) also indicated that testosterone infusion into coronary arteries in men with coronary artery disease induced vasodilation and that intravenous administration of testosterone reduced exercise-induced ischemic response in men with coronary artery disease. Testosterone's effect on vascular tone could be because of aromatization of testosterone to estradiol, as aromatase has been identified in the arterial wall (11). However, 17β-estradiol inhibits Ca^{2+} entry, whereas testosterone causes coronary relaxation by inhibiting other mechanisms in addition to Ca^{2+} entry (8). Furthermore, testosterone has been shown to exacerbate, whereas estrogen decreases, the vulnerability of lateral striatal artery to chemical hypoxia (25). The direct mechanism of testosterone action on arteries should also be taken into account.

Consistent with our in vivo study, testosterone was shown to exacerbate glutamate toxicity in an in vitro model. Toxic insults by glutamate in neuronal cell culture mimic a key component of ischemic brain injury. Microdialysis studies have shown that there is a severalfold increase in extracellular glutamate during global ischemia, beginning within 1–2 min (6, 24). There is a similar rise during focal ischemia, beginning within 2 min of MCAO (38). Furthermore, glutamate can cause both apoptosis and necrosis (28). In HT-22 cells, glutamate competes with cystine for uptake, leading to a reduction in glutathione, accumulation of reactive oxygen species, and ultimately cell death (35). The present study shows that testosterone treatment exacerbates glutamate toxicity to HT-22 cells, wheras 17β-estradiol treatment decreases the cells' susceptibility to glutamate toxicity, which provides us in vitro evidence to support our in vivo study. Although the deleterious effects of testosterone are only present at the micromolar level in vitro, which is thousands of times higher than peak physiological levels in reproductive males, physiological levels of testosterone exert damaging effects on ischemia-reperfusion injury in vivo.

It has been shown that in vivo treatment of postnatal rats with testosterone rendered cerebellar granule neurons less vulnerable to oxidative stress-induced apoptosis in vitro, which was associated with increases in catalase activity as well as in the activity of superoxide dismutase (1). However, the decreased susceptibility to oxidative stress induced by the postnatal treatment with testosterone was more likely due to an accelerated maturation with a consequent developmental age-dependent increase in the antioxidant defense (30). Effects of testosterone could be different in mature animals, as was shown with cerebral ischemia in our study. Testosterone treatment in vitro has also been shown to be neuroprotective for cerebellar granule neurons (2). As 17β-estradiol is also neuroprotective in cerebellar granule neurons (9), the neuroprotective effects of testosterone could be due to the conversion of testosterone into 17β-estradiol by aro-

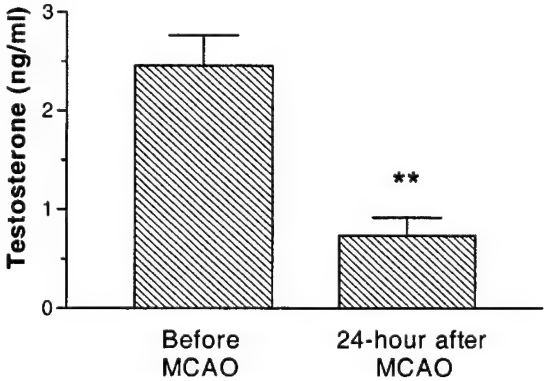

Fig. 5. Effect of ischemia-reperfusion injury on serum testosterone level in normal male rats ($n = 7$). Blood samples were taken before MCAO and 24 h after reperfusion. All blood samples were taken in the morning. Testosterone concentration was reduced to 0.73 ± 0.19 ng/ml at 24 h after reperfusion, compared with 2.46 ± 0.31 ng/ml before MCAO ($P < 0.01$). Values are means ± SE. $^{**}P < 0.01$ vs. testosterone concentration before MCAO.

matase. Furthermore, testosterone has been reported to attenuate neuronal death in mice in response to excitotoxins, which were blocked by aromatase (4).

In summary, the present data show that testosterone can increase neuronal toxicity and exacerbate ischemia-reperfusion injury. These results suggest that sex differences in the outcome after stroke may have resulted from both the protective effects of estrogens and the damaging effects of testosterone. Furthermore, acute depletion of testosterone provides neuroprotective effects on ischemia-reperfusion injury, which could be partially related to the amelioration of hyperemia during reperfusion.

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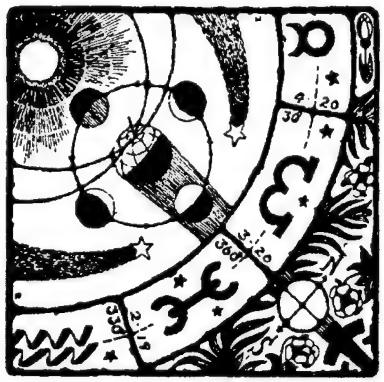

Appendix L

A NEUROENDOCRINE MECHANISM FOR TOLERANCE TO CEREBRAL ISCHEMIA-REPERFUSION INJURY IN MALE RATS

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Abstract

Testosterone has been shown to exacerbate cerebral ischemia-reperfusion injury, which suggests that the well-known stress-induced testosterone reduction could be a protective response. We hypothesized that stress-induced testosterone reduction contributes to ischemia tolerance in cerebral ischemia-reperfusion injury in male rats. In intact male rats, stress was induced by brief anesthesia at 6-hr before transient middle cerebral artery occlusion (MCAO). Testosterone levels were significantly decreased 6-hr after stress. Testosterone reduction was associated with a 50% reduction in cerebral lesion volume in the stressed animals. Further, the stress-induced cerebral ischemia tolerance was eliminated by testosterone replacement in castrated males. Immunohistochemical staining showed that androgen receptors were upregulated after cerebral ischemia-reperfusion injury and partially colocalized with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive cells in the parietal cortex and extensively colocalized in the caudate putamen. Heat shock protein 70 (Hsp70) and 90 (Hsp90) are involved in ischemia tolerance, and were not colocalized with TUNEL in the immunohistochemical staining, suggesting an anti-apoptotic role of Hsps. To determine the effect of testosterone on MCAO-induced Hsp70 and 90 expression, a testosterone replacement or withdrawal paradigm was used. Testosterone-replaced animals exhibited a decrease in Hsp expression, whereas testosterone withdrawal (mimicking the stress-induced testosterone suppression) normalized this deficit. In summary, stress-induced testosterone reduction contributes to ischemia tolerance in cerebral ischemia-reperfusion injury in males, which could be related to the loss of inhibition by testosterone of Hsp70 and Hsp90 expression.

Introduction

Preconditioning to ischemic tolerance is a phenomenon in which brief episodes of a subtoxic insult induce a robust protection against the deleterious effects of subsequent, prolonged, lethal ischemia. The beneficial effects of preconditioning were first demonstrated in the heart; but it is now clear that preconditioning can induce ischemic tolerance in a variety of organ systems including brain, heart, liver, small intestine, skeletal muscle, kidney, and lung (Murry et al., 1986; Moncayo et al., 2000; Serracino-Inglott et al., 2001). The profound protection induced by preconditioning makes this an attractive target for potential therapeutic development. Although first described over a decade ago, the mechanisms underlying the powerful protective effects of preconditioning to ischemic tolerance remain uncertain (Ishida et al., 1997). Understanding the mechanisms involved in cerebral ischemic tolerance has the potential to improve the prognosis of patients at risk for stroke.

The stimuli that constitute the preconditioning event are quite diverse, ranging from brief ischemic episodes, spreading depression or potassium depolarization, chemical inhibition of oxidative phosphorylation, and exposure to excitotoxins and cytokines (Bolli, 2000; Nandagopal et al., 2001). Other factors such as temperature and environment change could also contribute to the preconditioning (Rowland et al., 2000; Sammut et al., 2001). Testosterone levels are reduced after a variety kinds of stress (Norman and Smith, 1992; Tohei et al., 1997; Friedl et al., 2000; Morgan et al., 2000). Testosterone levels decrease after stroke, and concentrations of testosterone are inversely associated with stroke severity and 6-month mortality (Elwan et al., 1990; Jeppesen et al., 1996). Anesthesia can also decrease testosterone level (Sanhouri et al., 1990). The biological significance of this stress related testosterone reduction remains to be elucidated. On the other hand, testosterone has been shown to enhance cerebral ischemia-reperfusion injury

(Hawk et al., 1998). Furthermore, we have shown that testosterone depletion at 6 hours before stroke attenuates ischemia-reperfusion injury (Yang et al., 2002). The stress related testosterone reduction could be a neuroendocrine self-protective response and we hypothesized that stress induced testosterone reduction contributes to the cerebral ischemia tolerance against ischemia-reperfusion injury in males. In the present study, brief anesthesia was used as a stressor for preconditioning and effects of the stress-induced testosterone reduction on the subsequent cerebral ischemia-reperfusion injury were evaluated in a rodent middle cerebral artery occlusion (MCAO) model. Further, the role of androgen receptors, and heat shock protein 70 (Hsp70) and 90 (Hsp90) on apoptosis induced by cerebral ischemia reperfusion injury, and their relationship with the stress-induced testosterone reduction were also determined in the present study.

Materials and Methods

EXPERIMENTAL ANIMALS

Male Charles Rivers Sprague-Dawley rats (250g, Wilmington, MA) were maintained in laboratory acclimatization for three days prior to surgery. All animal procedures were approved by the University of North Texas Health Science Center Animal Care and Use Committee and University of Florida Animal Care and Use Committee.

MIDDLE CEREBRAL ARTERY OCCLUSION AND LESION DETERMINATION

To determine the effect of stress on subsequent ischemia-reperfusion injury, MCAO was induced in intact male rats. For the stress group, intact male rats were anesthetized by halothane and blood samples were collected via jugular vein 6 hours before MCAO. For the non-stress group, no anesthesia and blood sampling was performed before MCAO. To determine whether

stress induced testosterone reduction contribute to the stress induced cerebral ischemic tolerance, a castration and testosterone replacement paradigm was applied, that was a modification of the procedure which we previously used (Yang et al., 2002). Two days before MCAO, bilateral castration was performed under halothane inhalant anesthesia, and two 15mm long testosterone Silastic® pellets containing crystalline steroid were implanted subcutaneously immediately thereafter. Two days after castration and pellet implantation, stress was induced by brief anesthesia and pellet removal (testosterone depletion), or sham removal (testosterone replacement). Six hours after pellets removal or sham removal, transient MCAO was induced.

To determine the effects of androgen receptor antagonism on cerebral ischemia reperfusion injury, flutamide (20mg/kg) was administered subcutaneously in intact males, while control animals received vehicle. Transient MCAO was induced 2 hours later.

Ischemic stroke was induced in all animals by MCAO as previously described (Yang et al., 2002). Briefly, animals were anesthetized by intraperitoneal injection of ketamine (60mg/kg) and xylazine (10mg/kg). Rectal temperature was monitored and maintained between 36.5 and 37.5°C during the procedure. Blood was sampled from each animal via jugular vein immediately before stroke. With the aid of an operating microscope, the left common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were exposed through a midline cervical skin incision. The CCA and ECA were permanently cauterized. A 3-0 monofilament suture was introduced into the ICA via the ECA lumen, and advanced until resistance was encountered. The distance between the CCA bifurcation and the resistive point was about 1.9 cm. The middle cerebral artery was occluded for one hour and then suture was withdrawn for reperfusion. The CCA and ICA were coagulated and the skin incision was closed.

Each group of animals was decapitated 24 hours after reperfusion. Then the brain was harvested and placed in a metallic brain matrix for tissue slicing (Harvard Apparatus, Holliston, MA). Seven slices were made at 3, 5, 7, 9, 11 13 and 15mm posterior to the olfactory bulb. Each slice was incubated for 30 minutes in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) in physiological saline at 37 ° C, and then fixed in 10% formalin. The stained slices were photographed and subsequently measured for the surface area of the slices and the ischemic lesion (Image-Pro Plus 4.1, Media Cybernetics, Silver Spring, MD).

TISSUE PREPARATION AND IMMUNOHISTOLOGICAL STAINING

Animals were anesthetized with halothane at 24 hours after reperfusion and perfused transcardially with 0.9% saline, followed by 4% formalin. The brains were harvested and postfixed in 4% formalin. Tissue samples were embedded in paraffin and 7-μm coronal sections were cut by a microtome (Leica, Bannockburn, IL). The slides were deparaffinized, rehydrated and nonspecific binding sites were blocked by incubation of the slices in 10% normal goat serum and PBS-Tween for 1 hour at room temperature. For DAB staining, endogenous peroxidase activity was quenched by treating tissue sections with 3% hydrogen peroxide. The primary antibodies, Hsp70 (Sigma, St Louis, MO), androgen receptor and Hsp90 (Santa Cruz, Santa Cruz, CA), were diluted in the blocking solution and incubated with the specimens over night at 4°C followed by addition of biotinylated secondary antibodies. Streptavidin-peroxidase was then added and the presence of peroxidase was revealed by addition of the DAB substrate-chromogen solution (Zymed, South San Francisco, CA). For immunofluorescent staining, specimens were incubated with Alexa Fluor conjugated secondary antibodies (Molecular Probe, Eugene, OR) after incubation of the primary antibodies. For terminal deoxynucleotidyl transferase dUTP nick

end labeling (TUNEL) staining, sections were treated for 15 minutes with 20 μg/ml proteinase K in PBS then immersed in TdT buffer for 10 min, and then incubated with buffer containing TdT enzyme, and FITC labeled dNTP (Promega, Madison, MI) for 1hr at 37°C in a humidified chamber. The reaction was terminated by NaCL and sodium citrate for 15 min at RT. The sections were counterstained with DAPI, or receive further immunohistochemical staining. The light and fluorescent signal was visualized and photographed with a Nikon microscope with appropriate excitation/emission filter pairs.

TISSUE PREPARATION AND IMMUNOBLOTING

Animals were sacrificed at 2 or 24 hours after reperfusion. Cerebral cortices of each hemisphere were harvested separately in lysis buffer (50 mM Tris-base, pH7.4, 150 mM NaCl, 10% glycerol, 1mM EGTA, 1mM Na-orthovanadate, 5 μM $ZnCl_2$, 100 mM NaF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM PMSF, 1% Triton X-100) and homogenized by a Dounce homogenizer. The homogenate was centrifuged at 100,000 X g at 4°C for 30 min and the protein concentration of the supernatant was determined by Bio-Rad DC protein assay (Bio-Rad, Hercules, CA).

Equal amounts (40-50 μg) of samples were loaded onto 10% polyacrylamide gels and separated by SDS PAGE. The gels were electro-blotted onto nitrocellulose membranes. Immuno-detection of the protein of interest was carried out by first blocking the membrane in 5% nonfat dry milk in TBS-Tween (10 mM Tris-base, 150 mM NaCl, 0.2% Tween-20, pH 8.0), following by addition of the primary antibody: Hsp70 (Sigma, St Louis, MO) and Hsp90 (Santa Cruz, Santa Cruz, CA), which was diluted in the blocking solution. Excess antibody was removed by three washes of TBS-Tween. Membranes were incubated with a horseradish-peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse secondary antibody diluted 1: 5,000 in blocking

solution. The signal was developed by Enhanced Chemiluminescent method (Pierce, Rockford, IL), then imaged and analyzed by a Western Blot imagine system (UVP, Upland, CA).

SERUM TESTOSTERONE CONCENTRATION DETERMINATION

Serum was separated from blood cells by centrifugation and stored frozen (-20°C). Serum testosterone concentrations were determined using duplicate serum aliquots in a radioimmunoassay (Diagnostic Systems Laboratories, Los Angeles, CA).

DATA ANALYSIS

All data are expressed as mean ± SEM. Serum testosterone levels in each group were compared by one-way analysis variance (ANOVA) followed by Tukey tests. Lesion volume in each study was compared by student *t* test. The differences for each comparison were considered significant at $p<0.05$ level.

Results

Testosterone reduction contributes to the stress-induced cerebral ischemia tolerance in a middle cerebral occlusion model.

To determine effects of testosterone on the stress-induced cerebral ischemia tolerance, stress was induced by halothane anesthesia and blood sampling at 6 hours before MCAO. Testosterone level significantly decreased to less than 1 ng/ml at 6 hours after stress and no further reduction of testosterone level was detected at 24 hours after cerebral ischemia-reperfusion injury. In animals not subjected to stress, a modest reduction in testosterone levels was observed, because of the daily fluctuation of testosterone level (Simpkins et al., 1981)(Figure 1, A). A 50% reduction in lesion volume was associated with the stress induced at 6 hours before MCAO (Figure 1, B). In the castration and testosterone replacement paradigm, significant lesion volume reduction was observed when stress was applied in the absence of

testosterone (Figure 1, C). No beneficial effect of flutamide pretreatment on lesion volume was observed (Figure 1, D).

Upregulation of androgen receptor after cerebral ischemia-reperfusion injury.

Immunohistochemical staining of androgen receptors showed that expression of androgen receptors was increased at 24 hours after ischemia-reperfusion injury, both in the ischemic parietal cortex and caudate putamen area (Figure 2 and 3). The majority of cells in caudate putamen were TUNEL positive(Figure 3A), while fewer cell were TUNEL positive in the parietal cortex (Figure 3B). We observed full colocalization of TUNEL and androgen receptors in the caudate putamen (Figure 3A), but only partial colocalization in the ischemic parietal cortex (Figure 3B).

Testosterone inhibits expression of heat shock protein 70 and 90 after cerebral ischemia-reperfusion injury.

At 24 hours after cerebral ischemia reperfusion injury, immunohistochemical staining showed that both Hsp70 and 90 were expressed primarily in the penumbra of parietal cortex and did not colocalize with TUNEL, while very low levels of Hsp70 and Hsp90 were seen in the caudate putamen (Figure 4, A and B). To determine whether Hsp70 and Hsp90 expression was involved in the deleterious effects of testosterone on cerebral ischemia-reperfusion, Hsp70 and Hsp90 in cerebral cortex were detected by immunobloting. In testosterone replaced animals, Hsp70 and Hsp90 in the ischemia cortex were lower than that of testosterone depleted animals, at both 2 (Figure 5 A and C) and 24 hours (Figure 5 B and D) after reperfusion. On the contralateral non-ischemic cortex, no effects of testosterone replacement was seen at either time for either species of Hsp (Figure 5, A to D).

Discussion

Disruptions in homeostasis by either physiological or psychological stress induce a series of neural and endocrine adaptations known as a stress response, which is responsible for allowing the body to make the necessary physiological and metabolic changes required to cope with the homeostatic challenge. It is a common thought that the stress response is mediated by the activation of hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS) (Miller and O'Callaghan, 2002). Recently, functional cross-talk between the HPA and hypothalamic-pituitary-gonadal axis has also been demonstrated, which could contribute to the stress induced testosterone reduction (Viau, 2002). However, the significance of the well described stress-induced testosterone reduction is unclear.

Gonadal steroid hormones such as estrogens and androgens may affect various target tissues throughout the body including central nervous system. While the neuroprotective effects of estrogens have been well established (Green and Simpkins, 2000; McEwen, 2001; Nilsen and Brinton, 2002; Wise, 2002), the effects of androgen on nervous system are less clear. In the peripheral nervous system, testosterone may be a survival factor for axotomized motor neurons and promotes motor axon regeneration (Jones et al., 1999). In the central nervous system, both beneficial and deleterious effects of testosterone have been demonstrated (Ahlbom et al., 2001) (Yang et al., 2002). In the present study, transient anesthesia was used as a preconditioning stimulus in intact males and induction of cerebral ischemic tolerance was seen within 6 hours of preconditioning, an observation that is consistent with our previous report (Yang et al., 2002). The induction of cerebral ischemic tolerance was associated with a profound reduction of testosterone level. Further, the induction of cerebral ischemic tolerance was blocked by

testosterone replacement, supporting the hypothesis that stress-induced testosterone reduction is a neuroendocrine protective response that contributes to cerebral preconditioning in males.

The deleterious effect of testosterone on cerebral ischemia reperfusion injury could be related to its effects on aromatase activity. It has been shown that aromatase could play an important role in the mediation of the neuroprotective effects of the steroids (Azcoitia et al., 2001), which contribute to the cerebral ischemia tolerance. In normal rat brain, aromatase activity is present in neurons, whereas astrocytes and oligodendrocytes appear to have extremely low or no enzymatic activity (Steckelbroeck et al., 1999). Aromatase expression is induced in glial cells by different forms of brain injury (Garcia-Segura et al., 1999). Neurons from males possess a higher expression and activity of the enzyme than females and androgens negatively control expression/activity of aromatase in males (Negri-Cesi et al., 2001). Stress induced testosterone reduction could be neuroprotective by releasing its inhibitory effects on aromatase activity, hence, increasing the conversion of the remaining testosterone to estradiol in both neurons and glia.

Androgen receptors belong to the steroid receptor superfamily and play a pivotal role in the programming of male sexual differentiation and development (Janne and Shan, 1991). Immunohistochemistry studies indicated a wide distribution of androgen receptors in the central nervous system (Finley and Kritzer, 1999; Fodor et al., 2002). While the neuroprotective effects of lower concentration of testosterone appear to be androgen receptor dependent (Ahlbom et al., 2001; Hammond et al., 2001), several pieces of evidences in the present study indicate that the deleterious effects of testosterone may be androgen receptor independent. First, pretreatment with an androgen receptor antagonist in intact male rats did not produce protective effects against cerebral ischemia reperfusion injury in this transient MCAO model. Second, androgen receptors

were upregulated in the ischemic parietal and caudate putamen area at 24 hours after reperfusion, but were not extensively colocalized with cells undergoing apoptosis in the parietal cortex, the area of the brain protected by androgen reduction. While the significance of the upregulation of androgen receptors after cerebral ischemia reperfusion injury is not clear, the lack of complete colocalization of androgen receptors with TUNEL positive cells in the parietal cortex suggests that androgen receptors are not involved in initiating an apoptosis process. Further, in the caudate putamen where extensive androgen receptor TUNEL colocalization was seen, testosterone levels did not affect the size of the lesion caused by MCAO. As such, androgen receptor mediated processes do not appear to play a major role in the cell apoptosis cascade.

It is well known that cells respond to external stress in a highly conserved fashion. Among other mechanisms, preconditioning involves Hsps, which are induced by stressful stimuli and are thought to assist in the maintenance of cellular integrity and viability. Recent studies indicated that Hsps could directly interact with key components of the apoptotic machinery (Xanthoudakis and Nicholson, 2000; Christians et al., 2002). Hsp70 can act to usurp Apaf-1 and cytochrome C, thereby preventing maturation of caspase-9 by the apoptosome, hence inhibit activation of the downstream caspase cascade (Beere et al., 2000; Saleh et al., 2000). Hsp90 can also inhibit the formation of the functional apoptosome by sequestering Apaf-1 (Pandey et al., 2000). Consistently, in the absence of testosterone, increased expression of both Hsp70 and Hsp90 was seen in the cortical penumbra at 24 hours after cerebral ischemia-reperfusion injury, in cells not undergoing apoptosis, suggesting an anti-apoptotic effects of Hsps. Further, we have shown that testosterone exerts inhibitory effects on the expression of both Hsp70 and Hsp90 at 2 and 24 hours after cerebral ischemia-reperfusion injury. It appears, then, that acute stress induced

testosterone reduction could contribute to the cerebral ischemia tolerance by releasing the inhibitory effect of testosterone on Hsp70 and Hsp90 expression.

In summary, the present study indicated that stress induced testosterone reduction contributes to the cerebral ischemia tolerance against ischemia reperfusion, which provides the first *in vivo* evidences for a neuroendocrine mechanism for the cerebral preconditioning in males. The deleterious effects of androgen on cerebral ischemia-reperfusion injury appear to be androgen receptor independent and related to its suppression of expression of both Hsp70 and 90.

Acknowledgment

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Figure 1. Stress induced testosterone reduction contribute cerebral ischemic tolerance in males: **A.** Brief anesthesia induced a profound decrease in testosterone levels. In stress group, brief anesthesia was induced and blood was sampled (-6). 6 hours later animals were reanesthesized, blood was sampled (0) and transient MCAO was induced. 24 hours after reperfusion (24), blood was sampled again and brains were harvested for ischemia lesion analysis. The testosterone level was about 4 ng/ml in the morning and decreased to less than 1 ng/ml 6 hours after stress. No further reduction of testosterone was indicated at 24 hours after reperfusion. In the no stress group, blood was sampled and transient MCAO were induced in intact male rats. At 24 hours after reperfusion, blood was sample and brains were harvested for ischemia lesion analysis. Testosterone levels were about 2.5 ng/ml before MCAO and was less than 1 ng/ml at 24 hours after reperfusion. ** $p<0.001$ vs stress –6hr, $p<0.01$ vs no stress 0hr, * $p<0.05$ vs stress –6hr. **B.** Stress induced cerebral ischemic tolerance in males. Intact male rats were subject to stress, brief anesthesia, and transient MCAO was induced at 6 hours after stress. Stress group shown a 50% reduction of lesion size compared with non-stress group. * $p<0.05$ vs no stress group. **C.** Testosterone replacement eliminates stress induced cerebral ischemia tolerance. Male rats were subjected to castration and testosterone replacement two days before transient MCAO. For testosterone depletion (T depletion) animals, brief anesthesia was induced and testosterone pellets were removed two days later. Transient MCAO was induced 6 hours after pellet removal. For testosterone replacement animals (T), sham surgery was conducted and transient MCAO induced 6 hours later. $p<0.05$ vs T. **D.** No protective effects of pretreatment with the androgen receptor antagonist, flutamide, were seen in cerebral ischemia reperfusion injury. Intact male rats were treated with flutamide (20mg/kg) or vehicle 2 hours before MCAO, and then subjected to 1 hour MCAO and 24 hours reperfusion.

Figure 2. Upregulation of androgen receptors after cerebral ischemia reperfusion injury: Immunohistochemical staining of androgen receptors, with androgen receptors stained as brown and hemotoxylin counterstain for nucleus as blue. **a** and **b**: the ischemic and non ischemic parietal cortex, respectively. **c** and **d**: the ischemic and non ischemic caudate putamen, respectively.

Figure 3. Partial colocalization of androgen receptors and TUNEL positive cells after cerebral ischemia reperfusion injury: Androgen receptors (ARs) were stained red and TUNEL green. ARs were upregulated in the ischemic caudate putamen (A) and parietal cortex (B). More cells in the ischemic caudate putamen (**A**) are TUNEL positive compared with ischemic parietal cortex (**B**). TUNEL positive cells were partially colocalized with ARs in the ischemic parietal cortex, while all AR positive cells colocalized with TUNEL positive cells in the ischemic caudate putamen.

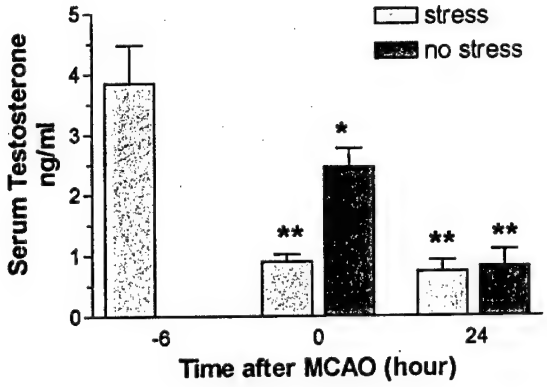
Figure 4. Lack of colocalization of Hsp70 and Hsp90 with TUNEL positive cells after cerebral ischemia reperfusion injury: Shown are immunohistochemical stainings of Hsp70 (**A**) and Hsp90 (**B**) as well as TUNEL at 24hr after transient MCAO. Both Hsp70 and Hsp90 were expressed in the parietal cortex and not colocalized with TUNEL. Very weak expression of both Hsp70 and Hsp90 was seen in the caudate putamen.

Figure 5. Testosterone suppresses Hsp70 and 90 expression after cerebral ischemia reperfusion injury: **A** and **B**: immunobloting and corresponding analysis of Hsp70 at 2 and 24hr after cerebral ischemia reperfusion injury, respectively, in the contralateral and ipsilateral cortex in

rats exposed to or depletion of testosterone. **C** and **D**: immunobloting and corresponding analysis of Hsp90 at 2 and 24hr after cerebral ischemia reperfusion injury, respectively in the contralateral and ipsilateral cortex in rats exposed to or depletion of testosterone. Suppression of both Hsp70 and 90 by testosterone was seen in the ischemic side at both time points. Animals from testosterone replacement (+) and depletion (-) group were sacrificed at 2 or 24 hours after reperfusion, and cerebral cortex were harvested and immunoblot for Hsp70 and 90. Immunoblot were repeated at least twice.

Figure 1.

A.


stress
no stress
Serum Testosterone ng/ml
5
4
3
2
1
0
*
**
**
**
-6
0
24
Time after MCAO (hour)

B

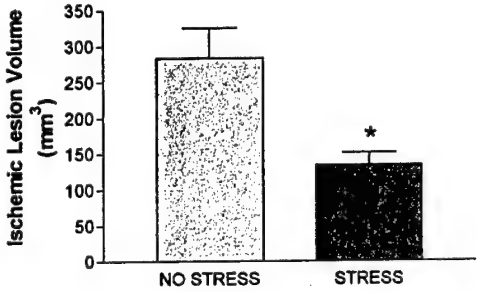
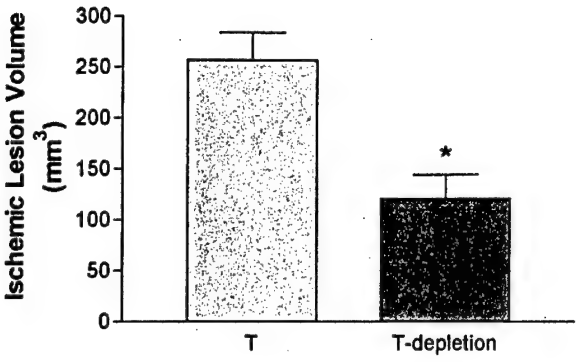

Ischemic Lesion Volume (mm3)
350
300
250
200
150
100
50
0
*
NO STRESS
STRESS

Figure 1.

C.


Ischemic Lesion Volume (mm3)
300
250
200
150
100
50
0
*
T
T-depletion

D.

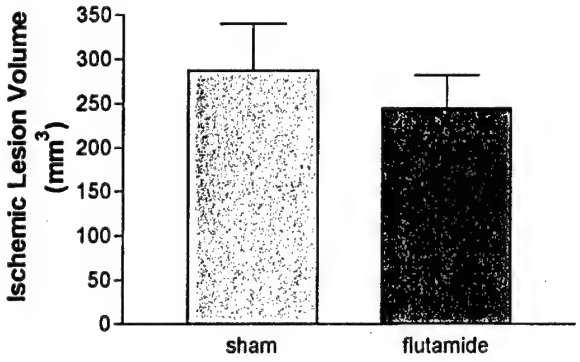

Ischemic Lesion Volume (mm3)
350
300
250
200
150
100
50
0
sham
flutamide

Figure 2.

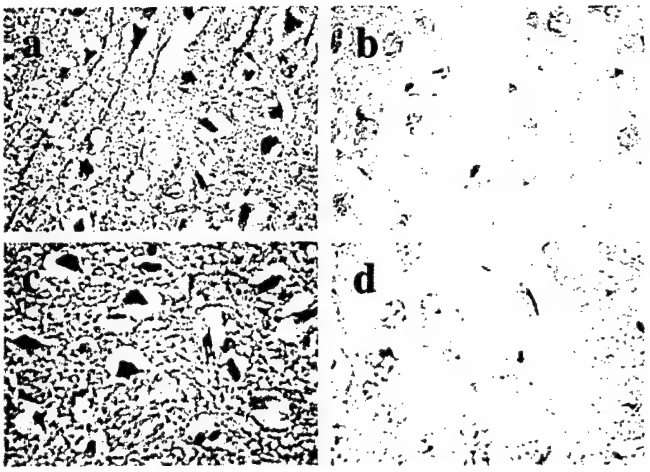
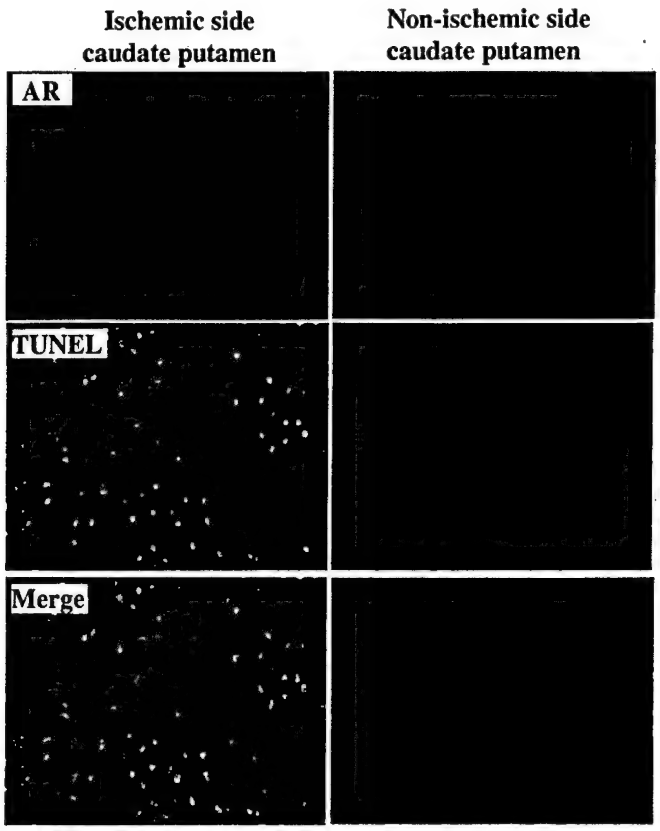


Figure 3.

A.


Ischemic side
caudate putamen
Non-ischemic side
caudate putamen
AR
TUNEL
Merge

B.

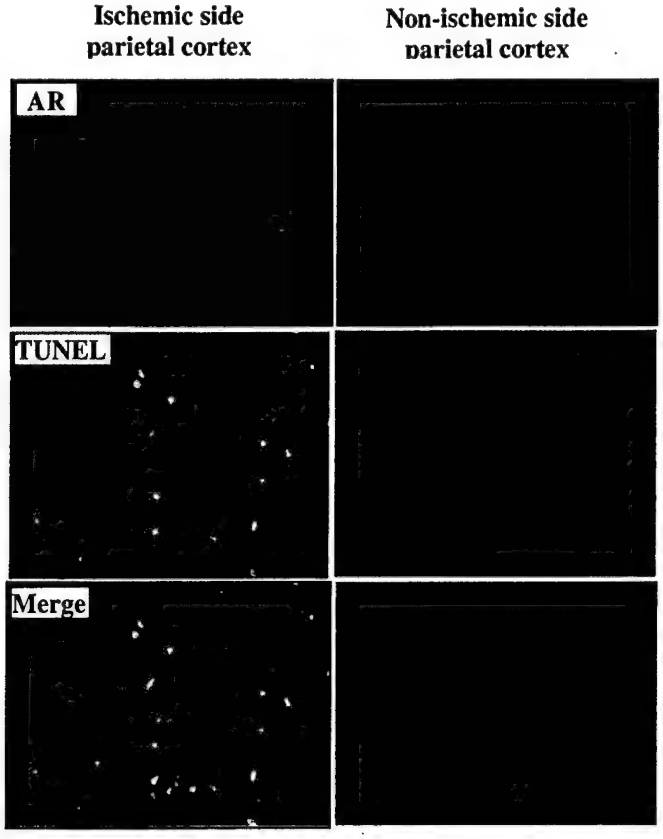

Ischemic side
parietal cortex
Non-ischemic side
parietal cortex
AR
TUNEL
Merge

Figure 4.

A.

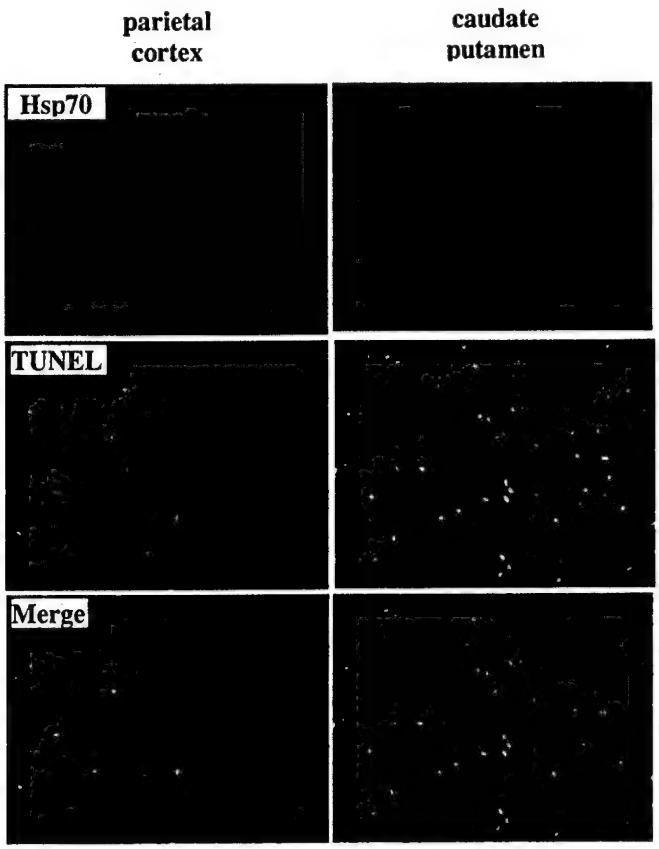
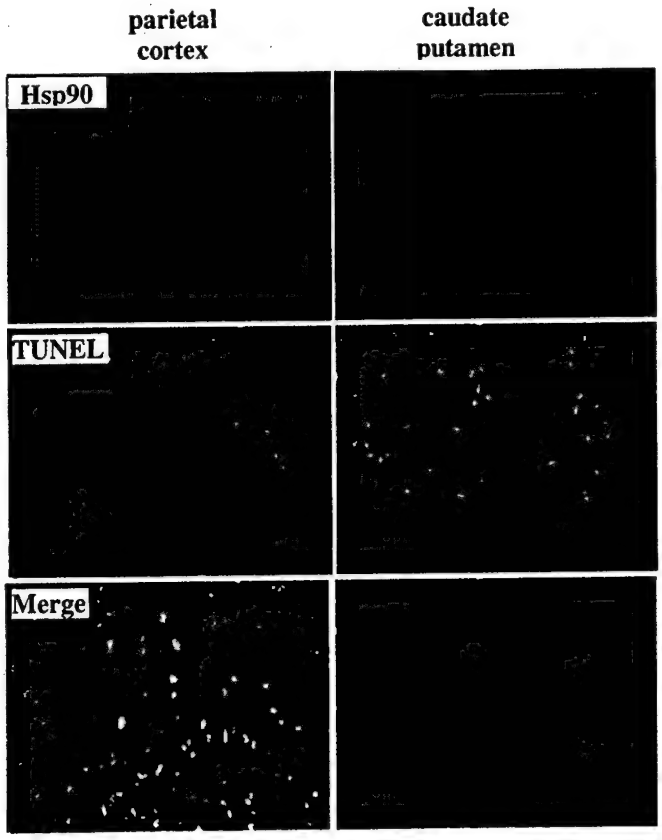


Figure 4.

B.



Appendix M

Estrogen Attenuates Nuclear Factor-Kappa B Activation Induced by Transient Cerebral Ischemia.

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Running title: Estrogen attenuates stroke induced NFκB

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Abstract:

The protective effects of estrogens have been well established in cerebral ischemia animal models, but the molecular mechanisms of this potent neuroprotective response are not well understood. Both *in vitro* and *in vivo* models indicate that in the central nervous system (CNS) and peripheral tissues, estrogen treatment reduces cytokine production and inflammatory responses. NFκB plays an essential role in the regulation of post-ischemic inflammation, which may have a detrimental role in the recovery from an ischemic stroke. We investigated the role of NF-κB in the survival of neurons *in vivo* and *in vitro*. We observed that a transient cerebral ischemia induced substantial apoptosis and inflammatory responses, including IκB phosphorylation, NF-κB activation, and iNOS over-expression. 17β-estradiol (E2) treatment had strong protective effects by reducing both neuronal apoptosis and inflammatory responses. Further, in HT-22 neuronal cultures, glutamate treatment caused severe neurotoxicity and inhibition of NFκB activation protected against this neurotoxicity. These findings provide evidence for a novel molecular and cellular interaction between the sex hormone and the immunoresponsive system. These studies also provide evidence that suppression of post-ischemic inflammation plays a critical role in estrogen-mediated neuroprotection.

Introduction:

Many epidemiological studies have shown that continued use of estrogen, and to a lesser extent, former use of estrogens, reduces stroke risk and mortality rate by an average of 50% [7-9]. Estrogens also may have a protective role in neurodegenerative diseases including Alzheimer's disease [10], Parkinson's disease [11], schizophrenia [12, 13]. Many other studies have also demonstrated the ability of estrogens to reduce oxidative damage, and protect cells in a variety of cell types and animal models [14]. Similar results have accumulated with the evaluation of estrogens in experimental ischemia. Results from our laboratory and others indicated a potent neuroprotective effect of estrogens in transient or permanent MCA occlusion models [15-19], as well as global ischemia [20, 21] and subarachnoid hemorrhage [22], indicating that estrogen treatment may be an effective pharmacotherapy for limiting the damage associated with a stroke.

Experimental middle cerebral artery occlusion (MCAO) is a widely used animal model to produce focal ischemic lesions in the rodents. This procedure causes a unilateral large ischemic area that typically involves the basal ganglion and frontal, parietal and temporal cortical areas [23, 24]. The ischemic lesion begins with the territory perfused solely by the MCA, and grows with time of occlusion [25]. The penumbral area around the core infarct is believed to result from the tissues that remain partially perfused by collateral circulation during the occlusion [24]. In the peri-infarct area, delayed neuronal death is associated with not only acute energy deprivation, but the consequent excitotoxicity, reactive oxygen species (ROS) production, and inflammatory responses [26].

Nuclear factor-kappa B (NFκB) is an inducible transcription factor that mediates the signaling of inflammation and cell stress in a number of cell types including neurons [27-31]. A variety of stimuli, including viral infection [32, 33], inflammatory cytokines [34, 35], hypoxia [36, 37] and oxidative stress [38, 39], can induce the activation of NFκB, which plays a pivotal role in the signaling of inflammation and cell stress. Active NFκB includes two subunits: a 50 Kd protein (P50) and a 65 Kd (p65 or Rel A) protein. Several isoforms and partner proteins have been identified, depending on cell types and environments of the stimuli [40]. The NFκB normally exists in the cytoplasm, is complexed to inhibitory binding proteins (IκB) and remains inactive. Upon stimulation, the IκBs are phosphorylated on Ser 32 and 36 by cytokine-responsive IκB kinases (IκK α or β), or other protein kinases. Phosphorylated IκBs are ubiquitinized by a ubiquitin ligase complex and subject to degradation by the 26S proteasome [41]. Dissociation and degradation of IκB induces the translocation of NFκB (P65 or P50) from cytosol to the nucleus where it binds to NFκB responsive elements (κRE), and facilitate the transcription of its responsive genes. Although the role of NFκB activation on cell survival during stress is highly controversial, inflammatory responses regulated by NFκB has an adverse effect in stroke, especially soon after the onset of an ischemic event [42-44].

Here we investigated the role of NFκB activation during cerebral ischemia-reperfusion, as well as the effects of a potent neuroprotective sex hormone, 17β-estradiol (E2). We provide evidence that NFκB activation, a pivotal regulator of post-ischemic inflammation, is strongly associates with delayed cell death during the reperfusion phase following transient ischemic induced by MCA occlusion. Estrogen treatment attenuates

the delayed cell death, as well as the activation of NFκB during post-ischemic reperfusion. This study demonstrates that estrogen-mediated neuroprotection is at least partially regulated through the reduction of post-ischemic inflammation, and reactive oxygen species (ROS) production.

Materials and Methods:

Animals:

Female Sprague Dawley rats were purchased from Charles Rivers (Wilmington, MA) and maintained in our animal facility in a temperature controlled room (22-25°C) with 12-hour dark-light cycles. All rats have free access to laboratory chow and tap water. All animal procedures were reviewed and approved by the University of North Texas Health Science Center Institutional Animal Care and Use Committee.

Cell cultures

HT-22 cells (ATCC, Rockville, MD), were maintained in DMEM medium (Invitrogen, Carlsbad, CA) containing 10% charcoal stripped fetal bovine serum (Hyclone, Logan, UT), and 20 μg/ml gentamycin under standard cell culture conditions (5% CO_2, 37°C). Cells used in these experiments were from passages 20-25 and inoculated at 3000-5000cells/well in 96 well-plates or 25,000-30,000 cells/well in 24 well-plates, 24 hours before experiments.

Ovariectomy:

Female rats were bilaterally ovariectomized using dorsal approach. Before surgery, animals were anesthetized with ketamine (60mg/kg) and xylazine (10mg/kg). A small cut was made through skin and muscle, the ovaries was externalized and removed. Ovariectomy was performed at least two weeks before of any further surgery.

Middle cerebral artery (MCA) occlusion:

Either oil vehicle or 100 μg/kg 17β-estradiol was administered by subcutaneous injection 2 hour before the onset of MCA occlusion. Animals were anesthetized by intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). MCA

occlusion was performed as previously described [15]. Briefly, the left common carotid artery, external carotid artery, and internal carotid artery were exposed through a midline cervical incision. A 3.0 mono-filament suture was introduced into the internal carotid artery lumen and gently advanced until resistance was felt. The suture was kept in place for 60 min and then withdrawn to allow reperfusion. The procedure was performed within 20 min, with minimal bleeding. Rectal temperature was monitored and maintained between 36.5 and 37.0 °C during the entire procedure. At desired time after the onset of reperfusion (2, 4, or 24 h), animals were decapitated and the brains were removed. The brains were then dissected coronally into 2-mm sections using a metallic brain matrix (ASI Instruments, Inc., Warren, MI), and stained by incubation in a 2% TTC in a 0.9% saline solution at 37 °C for 30 min, or fixed, embedded with paraffin, and sectioned to 8-10μm for immunohistochemical analysis.

Chemicals, antibodies and vectors:

2,3,5-Triphenyl-2H-tetrazolium chloride (TTC); sodium diethyldithiocarbamate hydrate (DDTC), and most other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Monoclonal anti-Rel A active subunit (Chemicon, Temecula, CA); anti-NOS2 (iNOS); anti-pIκB-α ($pSer^{32}$ specific) (Santa Cruz, Santa Cruz, CA); anti-β actin (Sigma St. Louis, MO) were used. Control-SEAP; NFκB-SEAP, P_{tal}-SEAP, DN-IκB were all purchased from (Clonetech, Palo Alto, CA).

TUNEL Staining:

TUNEL staining was performed according to the modified manufacturer instructions [45]. Formalin fixed, paraffin-embedded tissue sections were deparaffinizated with xylene, and rehydrated through a series of graded ethanol treatment

with a final wash in PBS. The sections were post-fixed in 4% paraformaldehyde for 20 min. Sections were then washed and treated for 15 minutes with 100 μg/ml proteinase K in PBS, then equilibrated with equilibration buffer for 10 min, and then incubated with buffer containing TdT enzyme, and FITC labeled dUTP (Promega, Madison, WI) at 37°C in a humidified chamber. The reaction was terminated by incubation in 2xSSC buffer for 15 min at room temperature (RT). The sections were then mounted withan anti-containingDAPI (Molecular Probes, Eugene, OR) or received further immunohistofluorescent staining. Positive control sections were immersed in DNase I solution for 10 min at RT, before equilibration in TdT buffer. The sections were observed under a fluorescent microscope with appropriate excitation/emission filter pairs.

Immunohistochemical Staining

Sections for immunohistochemical stainings were either stained with immunofluorescence, or HRP-conjugated polymer with DAB substrates (Zymed, South San Francisco, CA). Formalin-fixed and paraffin-embedded sections were cut into 8-10 μm thick slides. The slides received similar deparaffinization and rehydration treatments as described above for TUNEL staining. The sections were then blocked with 5% normal goat serum in 1% BSA for 30 min, then incubated with appropriate primary primary antibodies for 60 min at RT in a humidified chamber. After 3X rinsing with PBS, slides were incubated with species-specific secondary antibodies conjugated to Alexa^{488}, or Alexa^{594} (Molecular probes, Eugene, OR) for 40 min, The sections were then washed, counterstained, and mounted. The fluorescence was observed under a fluorescent microscope using excitation/emission specific for each fluorogen.

Transfections:

Before transfection, cells were plated in 24-well plates at 2.5 X 10^4 cells/well at least 24 hour before study. For transfection, Lipofectamine 2000 was used according to manufacturer's recommendation (Company, city, state). Briefly Lipofectamine 2000 was diluted into serum-free medium and incubated with appropriate amount of DNA for 20 min to allow complexes to form. Serum free medium containing transfection mix was first used for 4-5 hr and equal amount of growth medium containing 20% FBS were added to the wells until ready to assay.

SEAP-NFKB reporter Assay:

For transient transfection, Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used as described above. 120µl cell culture medium was removed and transferred to a microcentrifuge tube, centrifuged at 12,000g for 10 second to pellet any detached cell debris. 100 µl supernatant was transferred to a fresh tube and assayed. For the assay, 25 µl of each sample was placed into a separate well of a 96-well plate, and 25 µl of 1X dilution buffer was added and mixed gently. Samples were incubated for 30 min at 65°C to remove endogenous phosphatase activity. 97 µl of assay buffer was then added and incubated for 5 min at RT. Another 3 µl of the 1 mM MUP was then added and incubate for 60 min in the dark at RT. The results were measured with 96-well compatible fluorometer with the excitation/emission peaks at 360 nm/449 nm.

Immunoblotting analysis:

Tissues were harvested into protease and phosphatase inhibitor-containing lysis RIPA buffer: (1x PBS, 1% Igepal CA-630, 0.5% sodium deoxycholate, 1 mM Na_3VO4, 10 mM NaF, 10 µg/ml aprotinin, 1 µg/ml leupeptin, 1 mM PMSF). Protein concentration was measured with Bradford assay. Samples were boiled in SDS-PAGE loading buffer

or 5 min. 30–50 μg samples were loaded onto 8-16% gradient precast SDS-PAGE gels and separated based on molecular size. Prestained molecular size markers were used as molecular mass standards. The gels were then electroblotted onto polyvinylidene difluoride (PVDF) membranes. Immunodetection of the protein of interest was performed by first blocking the membrane in 5% nonfat dry milk in PBST (1XPBS, 0.02% Tween 20) followed by the addition of the primary antibody with specified dilution in blocking buffer. The PVDF membranes were then probed with HRP conjugated secondary antibodies. Positive signal was determined by enhanced chemiluminescence, and the specificity was determined by apparent molecular weight (MW) of the target protein.

Viability Assays:

Cell viability was quantified using calcein AM. Calcein AM is a cell permeable non-fluorescent compound, which can be quickly hydrolyzed by intracellular esterases to a highly fluorescent molecule, calcein, and easily quantified with a fluorescence microplate reader. This assay measures the total intracellular esterase activity as a marker of cell viability [46]. Cells were plated in 96-well plates at 5 X 10^3 cells/well, and exposed to various treatments. 24 hours after treatment, medium was discarded and cells were rinsed with PBS. 25 μM Calcein AM (Molecular Probes, Eugene, OR) in PBS buffer was added to each well. Plates were wrapped with aluminum foil and incubated for 20 minutes at 37 °C. Fluorescence was measured with excitation/emission at 495/538 nm. Wells without cells were used to determine background fluorescence.

Statistical Methods

The significance of differences among groups was determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. For

comaprison between two groups, student t tests were used. $P < 0.05$ was considered significant and each animal group consisted 4 to 7 replications. All values are expressed as mean ± SEM.

Results:

Transient focal ischemia rapidly induces apoptosis and nuclear NFκB translocation

As previously reported, neurons can die long after the stroke by apoptosis [47]. We examined the temporal and regional pattern of neuronal apoptosis, as well as NFκB activation in this experimental transient focal ischemia in rats (1-hour ischemia with desired time interval for reperfusion) using the mono-filament model [48].

To evaluate NFκB activation, we used an antibody raised against the nuclear localization signal of the NFκB subunit, Rel A [49]. This epitope is not accessible in the inactive form of NFκB in the cytoplasm, where it is masked by IκB proteins. As early as 2 hours after reperfusion, we observed evidence of apoptosis (positive TUNEL signal) and nuclear translocation of NFκB in the subcortical core area (Fig. 1), but not in the contralateral subcortex (Fig. 1). However, at this time point, many positive cells appear to be blood vessel epithelial cells or astrocytes. The activation of NFκB at 2hr was more widely spread than TUNEL staining (Fig. 1). The intensity of the stainings was relatively weak and occupied less than 5% of total cell numbers in the area, At 2 hr reperfusion, NFκB activation was already evident in the frontoparietal cortex, where TUNEL staining was negligible at this time (data not shown).

Both the intensity and density of TUNEL positive cells progressed with time. At 4 hr reperfusion, both TUNEL and active Rel A staining extended to the cortical area and both antigens appeared in cells that had neuronal morphology (data not shown). More than 15-30% cells in the subcortical area were stained positive for TUNEL and active Rel A (Fig. 1). At this time interval, the ischemic lesion was already evident based on H&E staining in the core region (data not shown). At 24 hr reperfusion, apoptosis dominated

the ischemic area, which involved the basal ganglion, frontoparietal cortex and some of the temporal cortex (Fig. 1). At 24 hr reperfusion, apoptosis and activation of NFκB, as well as its downstream effectors (iNOS), occupied more than 70% cells in the ischemic regions, where most cells would eventually be killed and removed, by 30 days after recovery (unpublished observation).

At 24hr reperfusion, NFκB translocation as well as activation of iNOS was evident in the ischemic region (Fig. 2B), while the contralateral cortex had little stainings (Fig. 2A). In the penumbra area, where the lateral ventricle separates the ischemic area from fully perfused tissue, the ventricle also separated positive Rel A and iNOS staining from the negative areas on the fully perfused side of the ventricular (Fig. 2C). Under higher magnification, most of the positive signals in the neocortex are identified in pyramidal neurons (Fig. 2B, D).

Delayed cell death is associated with NFκB activation.

In order to examine the relationship between NFκB activation and apoptosis, we performed colocalization studies. The temporal and regional pattern of NFκB activation and TUNEL stainings were strikingly similar. At 24 hr reperfusion, both were negligible in the contralateral brain section but were abundant in the ischemic cortex. Active Rel A signals were present in most TUNEL cells (Fig. 3), suggesting that NFκB activation is involved in the neuronal apoptosis induced by this transient ischemic event.

Over-activation of NFκB enhances the cytotoxicity in neuronal cell lines induced by glutamate.

As *in vivo* studies provided only limited evidence about the function of NFκB in neuronal apoptosis, we further examined the role of NFκB in a neuronal cell culture

system. HT-22 cells, a transformed hippocampal mouse neuronal cell line, are sensitive to high concentrations of glutamate, through a non-NMDA receptor mediated oxidative mechanism [50]. We confirmed the activation of NFκB induced by high glutamate concentrations by transfecting a reporter gene, which contains four tandem copies of the NFκB consensus sequence fused to a TATA-like promoter (P_{TAL}) (Clontech, Palo Alto, CA). PTAL-SEAP, that contains only the TATA-like promoter (P_{TAL}) region, induced a basal level of SEAP activity, while glutamate induced nearly five-fold increase in SEAP activity, and this activation was suppressed by a co-transfection of dominant negative IκB vector DN-IκB (Fig. 4A). The dominant-negative IκB vector, DN-IκB, contains two mutations from serine to alanine at residues 32 and 36, which prevents its phosphorylation on these specified residues, thereby blocking the dissociation and degradation from the NFκB subunits. DN-IκB was highly protective against this glutamate induced neurotoxicity (Fig. 4B).

Further, we examined cell viability in the presence of DETC, a NFκB inhibitor. DETC is reported to inhibit the activity of NFκB and some cytokine production [51, 52]. DETC was toxic to HT-22 cells at high concentrations (data not shown). However, 5μM DETC protected HT-22 cells from glutamate induced cell death (Fig. 4C).

Estrogen treatment reduces NFκB activation, as well as apoptosis in MCAO rats.

Previous studies have demonstrated a potent neuroprotective activity of estrogens in a variety of cell types and animal models [14]. We studied the effects of estrogens on the regulation of this pivotal inflammatory response, NFκB activation, its upstream regulator (IκB), its downstream effector (iNOS), as well as apoptosis during this ischemia-reperfusion process. In this study, we found that E2 treatment caused profound

inhibition of NFκB activation induced by transient cerebral ischemia. Fig. 5 shows the representative photomicrographs of the TUNEL staining, immunostainings of inflammatory signaling intermediate proteins, which includes Active Rel A, iNOS and $pI\kappa B^{ser32}$, in ovariectomized and estrogen treated animals (Fig. 5). E2 caused a profound reduction of both TUNEL staining and the activation of each of the inflammatory intermediate proteins.

To quantitatively assay these inflammatory markers, we used immunoblotting with a variety of antibodies of protein extracts from ischemic cortex of ovariectomized and E2 treated rats; as well as the contralateral cortex from ovariectomized rats. For both phospho-specific IκB and iNOS, there were negligible protein in the contralateral cortex (Fig. 6A, B), similar to our observation using immunohistochemical assessment (Fig.1). There was a large induction by ischemia/reperfusion of phosphorylation of IκB and of iNOS expression, both of which were significantly suppressed by E2 treatments (Fig. 6A, B). Total protein levels for the p65 subunit of NFκB (Rel A) was not affected by cerebral ischemia (Fig. 6A, B). Total protein levels, however, do not reflect its activity, as the denaturing gel releases inactive Rel A from its inhibitory protein, IκB.

Discussion:

The present study made several important observations. First, NFκB is rapidly activated during the reperfusion phase of ischemia/reperfusion. Second, this activation occurs in basal ganglion and cortical neurons that are in the process of undergoing apoptotic cell death, as evident by colocalization of activated NFκB in TUNEL positive neurons. Third, a NFκB activity regulator, iκB, and a down stream effector, iNOS are rapid activated during ischemia/reperfusion. Finally, E2 treatment markedly blunts all of these responses to ischemia/reperfusion. Collectively, these results suggest that activation of NFκB is a critical component in neuronal apoptosis that occurs during cerebral ischemia/reperfusion.

Activation of NFκB plays a pivotal role on the regulation of inflammatory and oxidative responses. NFκB regulates the expression of numerous genes involved in immunity, inflammation, oxidative damage and apoptosis in a variety of cell types [53-56] and therefore may be a good therapeutic target for the treatment of stroke. Cell culture models indicate NFκB activation plays a protective role in oxidative damages apparently up regulating a number of pro-survival factors [57-60]. On the other hand, NFκB activation is neurotoxic in ischemic stroke *in vivo* models. Proteasome inhibitors (which also inhibit NFκB activation), including MLN519 and NAC, are highly protective against ischemic stroke [61, 62]. Further, a transgenic model where the p50 subunit of NFκB is knocked out, showed a smaller-sized cerebral infarcts, compared with wild type [49]. Our observation that inhibition of NFκB activation through genetic manipulation or pharmacological inhibition effectively prevented cell death *in vitro*, supports the

conclusion that NFκB activation is a neurotoxic mediator of pro-oxidant and pro-inflammatory signals.

NFκB plays a critical role in the inflammations, by regulating inflammatory mediators such as IL-1, IL-6, IL-8, inducible nitric oxide synthase (iNOS), ICAM 1, VCAM, and E-selectin [40]. All these inflammatory mediators are up-regulated during the reperfusion stage of ischemic tissues and are thought to signal and facilitate neutrophil invasion of the brain tissues [61, 66]. This invasion can lead to significant cellular damage and neuronal death. In our studies, NFκB activation was initiated as early as two hours after an ischemia/reperfusion stroke and dominated the ischemic region after 24-hour reperfusion. Accordingly NFκB activation was accompanied with iNOS over-expression and apoptosis. This observation is consistent with previous findings of elevations in key cell adhesion molecules and inflammatory cytokines after stroke [67-69]. NFκB activation was widespread in the peri-infarct area and highly colocalized with the apoptotic marker, TUNEL, suggesting that NFκB activation occurs in neurons destine to die by apoptosis. This NFκB activation in these dying neurons is likely due to high concentrations of reactive oxygen species (ROS), a known activator of NFκB. Additionally, activation of NFκB enhances cytokines and chemokines, causing microglia activation and leukocyte infiltration into the ischemic site, thereby contributing to the secondary injuries during post-ischemic recovery. Collectively, this suggests that inhibition of NFκB activation is neuroprotective and can effectively limit neuronal loss from ischemia/reperfusion injuries.

In our studies, a single pharmacological dose of 17 β-estradiol was sufficient to suppress transient ischemia-induced NFκB activation, as well as to reduce apoptotic

neuronal death. These observations are consistent with many previous findings. Estrogens had been demonstrated as potent neuroprotective agents against ischemic stroke **[15, 22].** Estrogen treatments have been shown to reduce oxidative damage, and protect a variety of neurons *in vitro* and in animal models [14]. Further, epidemiological studies indicate the benefit of estrogen in stroke occurrence and recovery [7-9]. Both our laboratory and others reported a potent neuroprotective effect of estrogens in transient or permanent MCA occlusion models [15-19], as well as global ischemia [21] and subarachnoid hemorrhage [22]. It appears that estrogens may be an effective pharmacotherapy for the treatments of an ischemic stroke.

The mechanism of this potent protection by estrogens is still not completely defined, but the present report suggests that suppression of activation of NFκB contributes to the potency of its effect *in vivo*. Estrogens regulate the transcription of a number of genes by binding to estrogen receptors that act as transcriptional factors at the estrogen responsive element (ERE). Estrogen receptors can also form heterodimers with other transcriptional factor and affect transcription of genes that do not contain ERE [70]. Many novel, non-transcriptional properties of estrogens have been demonstrated, including antioxidant property, phosphorylation of critical signaling proteins, modulation of NMDA receptors and effects on nitric oxide synthesis [71-75]. Estrogens are reported to induce proteinase inhibitor 9 (PI-9), which inhibits caspase-1 (IL-1beta converting enzyme) and granzyme B, thereby regulating production of the pro-inflammatory cytokine IL-1beta and susceptibility to granzyme B-induced apoptosis [76]. E2 is reported to antagonize NFκB activation in ovariectomized mice fed an atherogenic diet

[3]. On the other hand, an estrogen antagonist, Tamoxifen, enhances TNF-alpha induced MnSOD expression possibly through modulation of NFκB dimerization [77].

In summary our studies indicated that estrogen-induced suppression of NFκB protects neurons from this ischemic damage by reducing inflammatory activation and apoptosis. This indicates that NFκB is an attractive target for post-ischemic inflammation control in stroke therapy.

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Figure legends:

Figure 1: The temporal pattern of the activation of NFκB and apoptosis induced by transient cerebral ischemia/reperfusion. Representative photomicrographs of TUNEL (left column), and active Rel A (right column). All photomicrographs are of the subcortical basal ganglion area (referred to as core area). All animals received MCAO for 1 hr and reperfusion for the indicated time. Scale bar indicates 100 μm.

Figure 2: The regional pattern of the NFκB activation, iNOS expression and apoptosis at 24 hr of reperfusion. Consecutive sections were stained with iNOS (left column), active Rel A (middle column), and TUNEL (right column). (A): Contralateral cortical area; (B) Ipsilateral cortical area; (C): Sub-ventricular area, where lateral ventricle separates the ischemic and fully perfused brain regions. * indicates the location of lateral ventricle; Arrow indicates the positive DAB signals. (D) High magnification staining of cortical area. Scale bar indicates 100 μm.

Figure 3: The colocalization of active Rel A with TUNEL staining at 24 hr of reperfusion. Blue indicates DAPI nuclear counter-stain, which labels nuclei; green colors indicate positive TUNEL staining; red colors indicated activated NFκB positive cells. The last column shows the merged pictures. The arrow indicates some of the colocalized signals of TUNEL and active NFκB in nuclei. Scale bar indicates 100 μm.

Figure 4: Over-activation of NFκB induces cytotoxicity in neuronal cell lines. (A): HT-22 cells were transfected with control-SEAP, pTAL-SEAP, NFκB-SEAP, or NFκB-

SEAP+DN-IκB 24 hr before glutamate treatments. A final concentration of 20mM glutamate was added to the culture medium, and the supernatant was assayed for SEAP activity. SEAP activity was normalized to the cell numbers for each group. Data are expressed as mean ± SEM for n= 4 (B): HT-22 cells were transfected with vehicle vectors or DN-IκB and cell viability was measured with Calcein AM assay. Data are expressed as mean ± SEM for n= 6 (C): HT-22 cells with or without 5μM DDTC were treated with different concentrations of glutamate. Depicted are mean ± SEM for n= 6. Symbols indicate a statistically significant difference between the indicated groups at * $P<0.05$; ** $P<0.01$; *** $p<0.001$.

Figure 5: Estrogen treatment attenuates TUNEL staining as well as other post-ischemic inflammatory markers. Representative photomicrographs of TUNEL staining; and immunofluorescence of active Rel A, iNOS, and p-IκB taken from similar regions in the frontoparietal neocortex of ovariectomized (left) and 17β-estradiol treated (right) female rats. All animals received 24 hr reperfusion after a 1 hr MCA occlusion.

Figure 6: Immunoblotting analysis of NFκB-related gene expression in response to cerebral ischemia and estrogen treatments. (A): Western blot analysis of pIκB, iNOS, NFκB P65 subunit and the loading control, β-actin. Lanes 1-3 were from the ipsilateral cortex of ovariectomized (Ovx) animal, lanes 4-6 were from the ipsilateral cortex of Ovx+E2 rats, and lanes 7-9 were from the contralateral cortical Ovx rats. Each lane represents an individual experimental animal. (B) Densitometric analysis of immunoblots

in A. Symbol indicates significant difference between the indicated group and the ipsilateral cortex of ovariectomized animal at *$p<0.05$; **$p<0.01$.

Figure 1

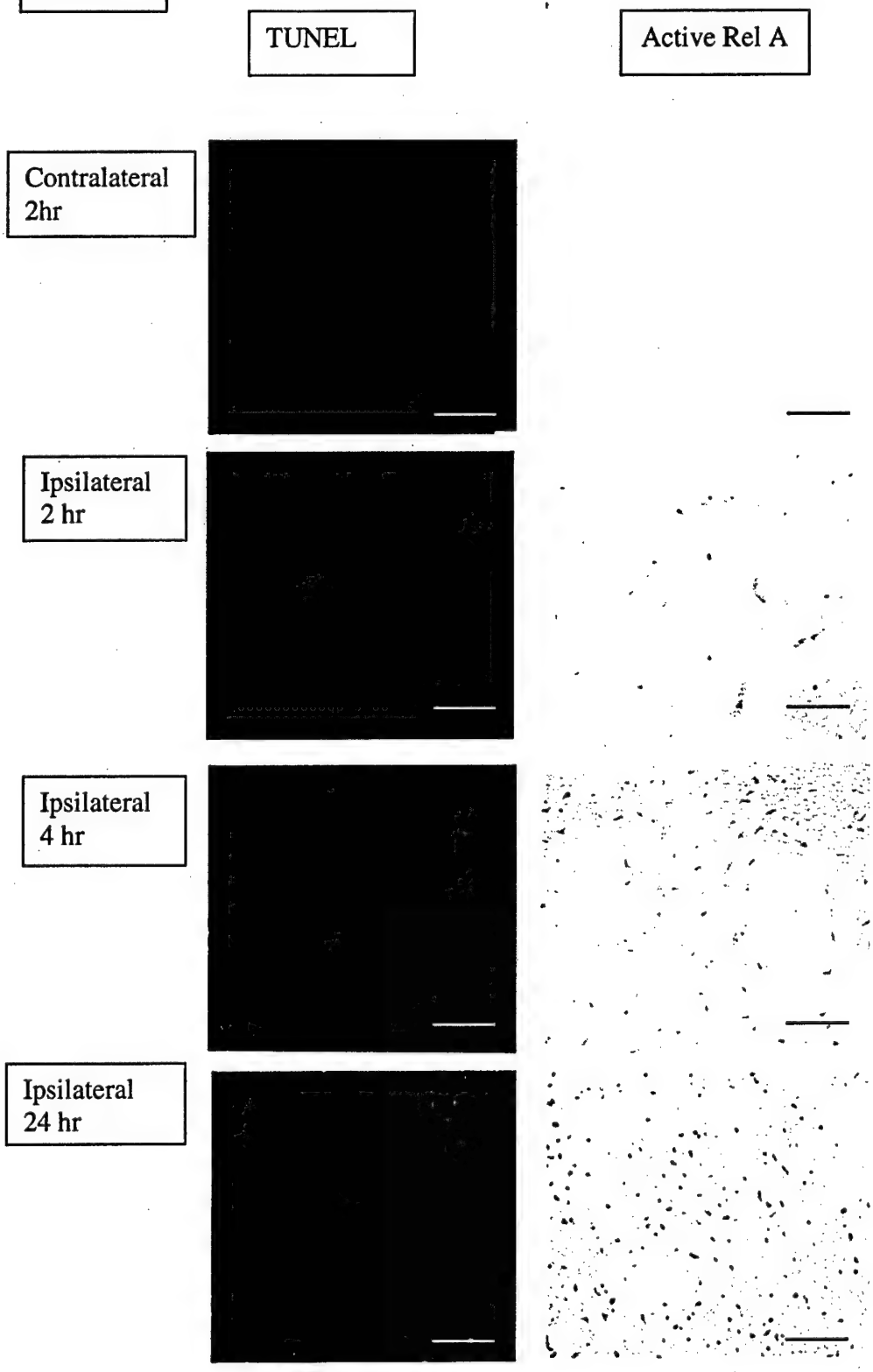

TUNEL
Active Rel A
Contralateral 2hr
Ipsilateral 2 hr
Ipsilateral 4 hr
Ipsilateral 24 hr

Figure 2

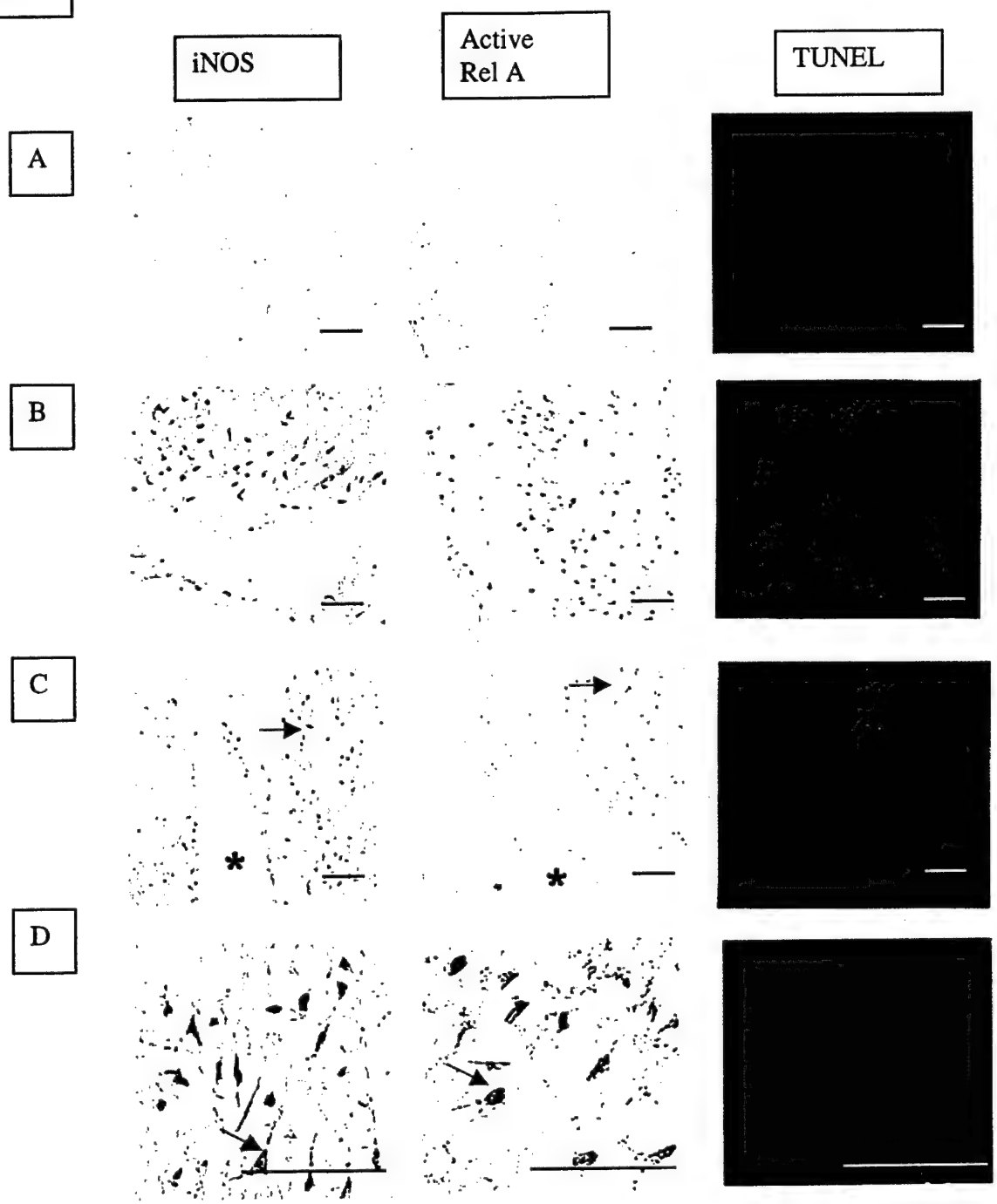


Figure 3

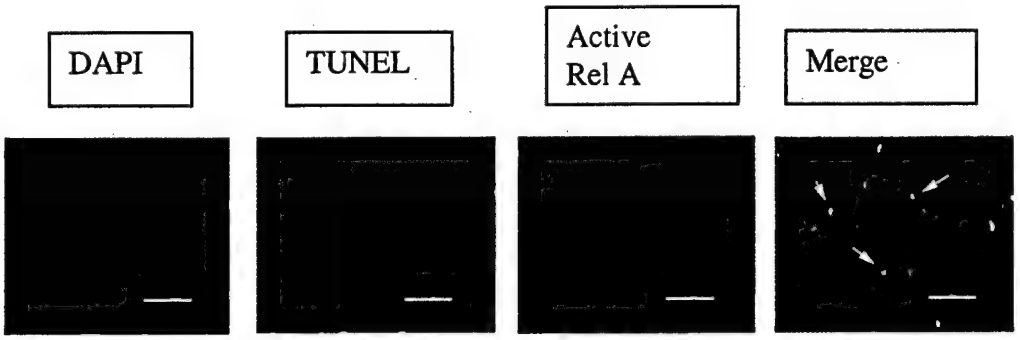
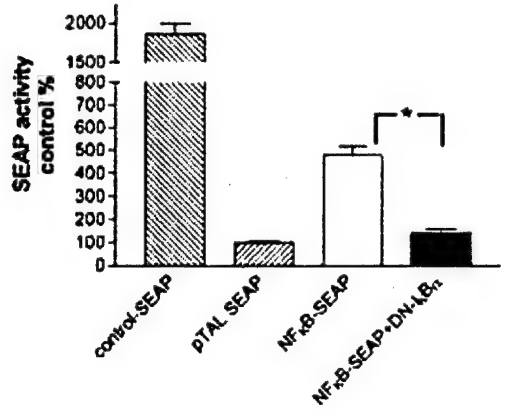
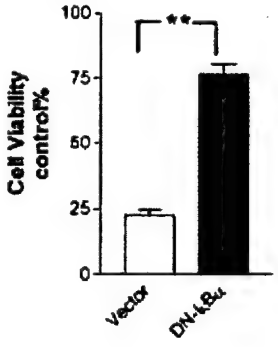


Figure 4

A


SEAP activity control %
2000
1500
800
700
600
500
400
300
200
100
0
*
control-SEAP
pTAL SEAP
NFκB-SEAP
NFκB-SEAP+DN-IκBα

B


Cell Viability control%
100
75
50
25
0
**
Vector
DN-IκBα

C

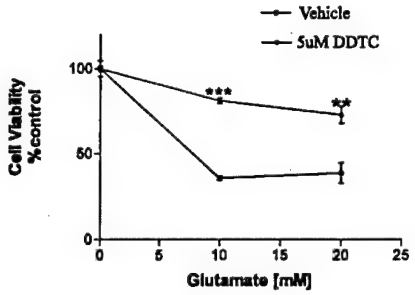

Vehicle
5uM DDTC
Cell Viability %control
100
50
0
***
**
0
5
10
15
20
25
Glutamate [mM]

Figure 5

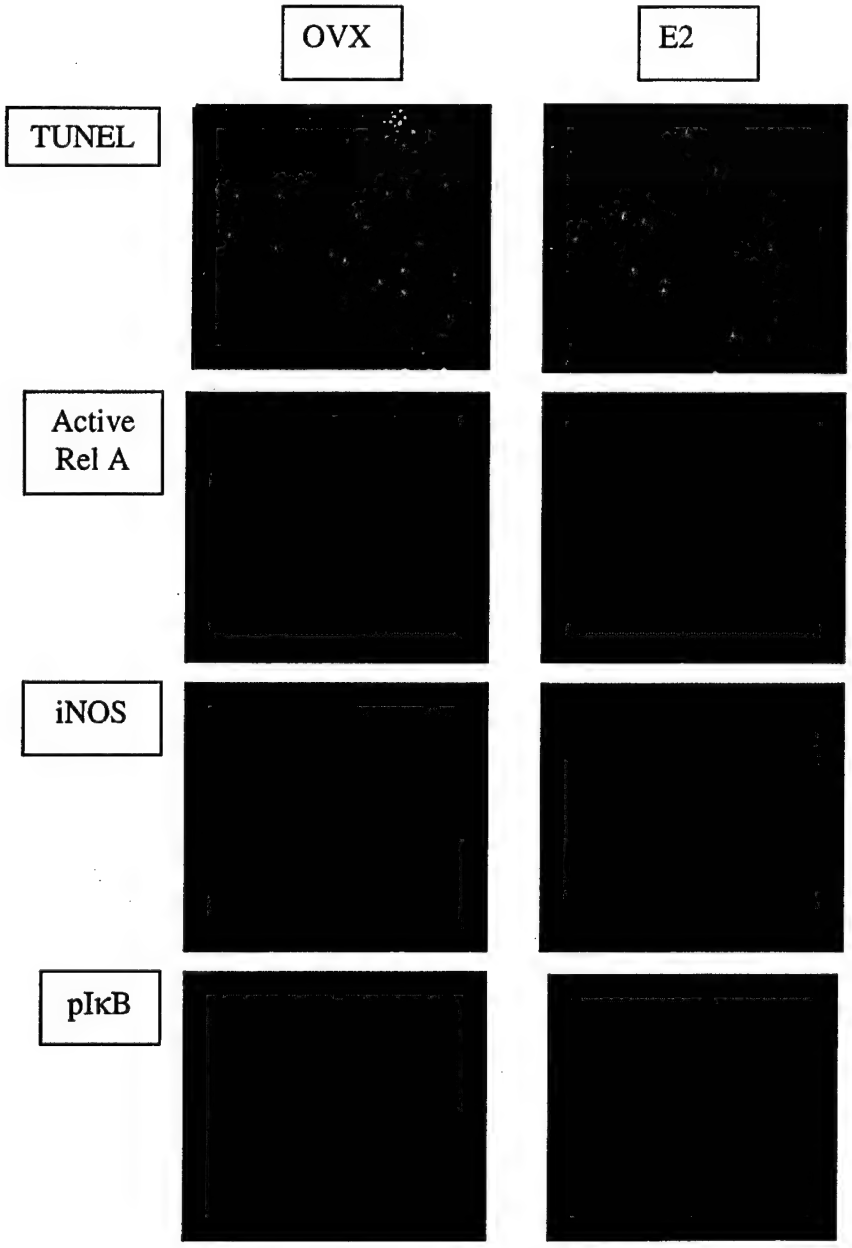
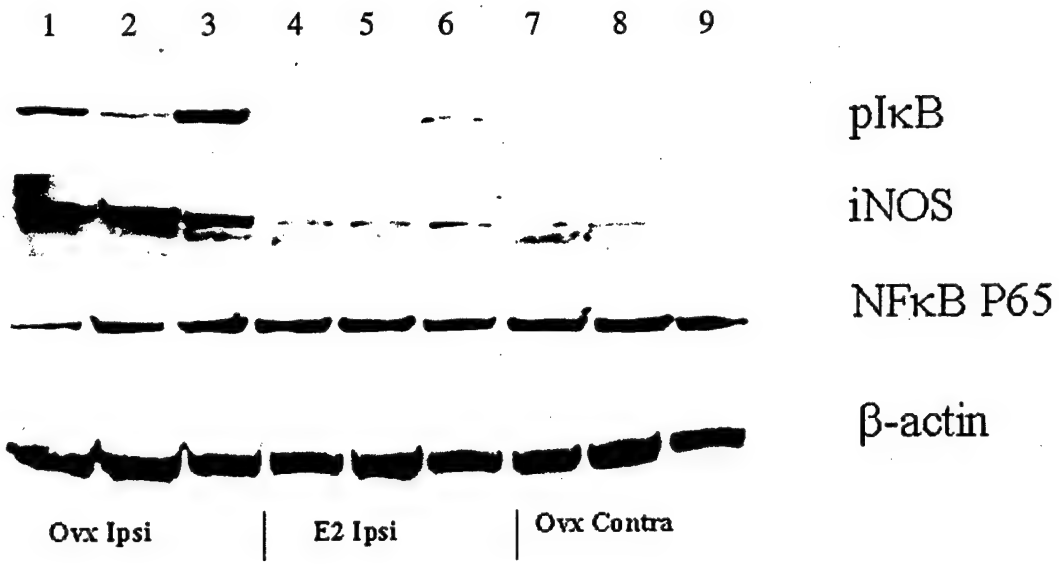
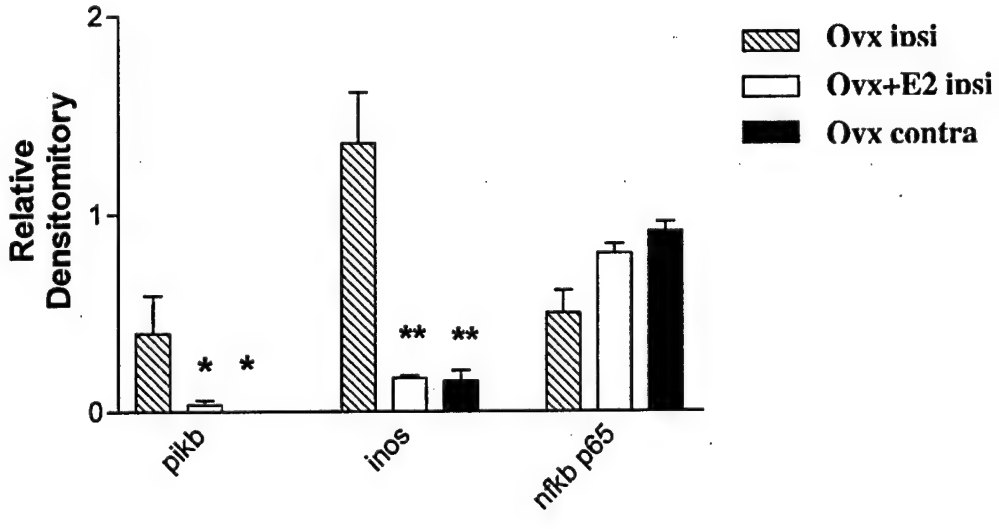


Figure 6

A


1 2 3 4 5 6 7 8 9
pIκB
iNOS
NFκB P65
β-actin
Ovx Ipsi
E2 Ipsi
Ovx Contra

B


Relative Densitomitory
2
1
0
* *
** **
pikb
inos
nfkb p65
Ovx insi
Ovx+E2 insi
Ovx contra

Appendix N

Cerebral Ischemia Induces Alzheimer's Disease-Like Neurofibrillary Tangles and Aberrant Neuronal Mitosis

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Abstract:

Aberrant mitosis occurs in many tauopathy-related neurodegenerative diseases and is believed to precede the formation of neurofibrillary tangles (NFTs). Herein, we report for the first time that transient cerebral ischemia induces aberrant mitotic proteins and neurofibrillary tangles in post-mitotic neurons of in rats. The temporal pattern of the appearance of these neuropathological markers for Alzheimer's disease (AD) following an ischemic event indicate that the early initiation of apoptosis precedes and is potentially integrated with signals for subsequent aberrant mitosis and neurofibrillary tangles. The female sex steroid and potent neuroprotective agent, 17 β-estradiol, reduced ischemia-reperfusion induced cerebral damage, and the subsequent aberrant mitosis and tauopathies. These results provide a neuropathological basis for the higher prevalence of dementia in stroke patients and support the hypothesis that apoptosis and aberrant mitosis are integrated pathological events in neurons that may play a critical role in the developments of AD neuropathology.

Introduction

The prevalence of dementia in ischemic stroke patients is nine-fold higher than controls at 3 months (Tatemichi et al., 1992) and 4-12 times higher than in controls at 4 years after a lacunar infarct (Loeb et al., 1992). Many of these dementias developed progressively and cerebral damage is believed to be the direct cause of cognitive decline of only half of these cases (Tatemichi et al., 1994). Alzheimer's disease (AD) is the most prevalent dementia (Pasquier and Leys, 1997) and shares common neuropathology features with stroke. Amyloid angiopathy can lead to cerebral hemorrhage (Vinters, 1987), Alz-50-immunoreactive granules are found around cerebral infarction after a stroke(Ikeda et al., 2000), amyloid precursor protein (APP) accumulates following transient focal ischemia (Shi et al., 2000), and ApoE4 is a genetic risk factor for both AD and stroke (Contois et al., 1996).

Neurofibrillary Tangles (NFTs), whose major component is hyperphosphorylated tau (Goedert, 1993), are observed in many neurodegenerative diseases (Spillantini et al., 1998; Spillantini and Goedert, 1998). Aberrant mitosis in differentiated neurons involves the activation of many cyclin-dependent kinases. Cdc2 and cyclin B1, as well as many other mitotic markers, are elevated in nearly all tauopathy-related neurodegenerative diseases (Husseman et al., 2000). These protein kinases regulate cell cycle progression into mitosis in developing and proliferating cells, and may lead to apoptosis in terminally differentiated cells (Enokido et al., 1996; Nuydens et al., 1998). In AD and other diseases tauopathies, induction of cdc2 and cyclin B1 as well as aberrant DNA replication is observed in neurons containing NFTs (Yang et al., 2001). However, completion of successful nuclear divisions in differentiated neurons has never been observed. These

inappropriately induced mitotic protein kinases can phosphorylate tau, modify other cytoskeletal proteins, and further induce apoptosis in terminally differentiated cells (Yen et al., 1995).

Recent studies reveal an intricate interaction between apoptosis, mitosis and formation of NFTs in post-differentiated neurons. Activation of programmed cell death is observed in AD brains. Apoptosis, as indicated by TdT-mediated dUTP Nick-End Labeling (TUNEL) (Katsuse et al., 2001) positive neurons and activation of neuronal caspase-3 (Stadelmann et al., 1999) is widespread in AD brains. Survivin, a member of the Inhibitor of Apoptosis Protein (IAPs) family is induced under stress, as well as in the G2/M phase of the cell cycle and binds to spindle microtubules (Uren et al., 1999). Up-regulation of cyclin D1, as well as other cell cycle induction marker, is observed during apoptosis in post-mitotic neuronal cultures (Shirvan et al., 1997; Sakai et al., 1999; Stadelmann et al., 1999) and cell cycle inhibitors are protective against apoptotic stimuli in neurons (Farinelli and Greene, 1996). Finally, there is evidence that mitotic events may contribute to the formation of neurofibrillary tangles (NFT) (Vincent et al., 1996; Husseman et al., 2000).

In the present study, we assessed the temporal pattern and colocalization of markers of apoptosis, mitotic protein activation and NFTs in a rodent model for cerebral ischemia. We report for the first time, the induction of NFTs in a non-transgenic animal model and that apoptotic signals precede both mitotic protein activation and formation of NFTs. These results suggest that therapies directed at apoptosis may be able to prevent the sequelae of neuropathological events seen in AD.

Result

Cerebral ischemia-reperfusion rapidly induces apoptosis and subsequent mitotic events

Middle cerebral artery occlusion (MCAO) is a widely used ischemic stroke model (Bremer et al., 1975). This *in vivo* model for neuronal death has the distinct advantage that apoptosis is rapidly induced and synchronized in a large number of neurons (Li et al., 1997). Initially, we examined the effects of transient cerebral ischemia on apoptosis and the expression of mitotic markers. As early as 2 h after the initiation of reperfusion following a 1h MCAO, positive TUNEL staining appeared in the core ischemic region within the basal ganglion, but no mitotic markers were observed in the neurons in the ischemic region (Fig. 1a). At 4h of reperfusion, the brain areas and number of cells displaying TUNEL positive staining had increased. At the same time, expression of mitotic markers, cyclin B1 and MPM-2, appeared in the ischemic core (Fig. 1b&d), and these markers extended into parts of the cortex around the infarct area (data not shown). Cyclin B1 is the activator of cdc2 protein kinase, and accumulates during mitosis(Fang and Newport, 1991), and the MPM-2 monoclonal antibody recognizes a set of M-phase specific phosphoproteins, most of which are the substrates of cdc2/cyclin B1 (Davis et al., 1983). No neurofibrillary tangles were detected at 4h of reperfusion after MCAO in either the ischemic region or the contralateral brain side (data not shown).

Delayed cell death induces aberrant mitotic protein.

The time course of the ischemia-reperfusion induced cellular changes was studied. We found that the density and staining intensity of TUNEL positive cells progressed with time and was evident throughout the infarcted brain region at 24h of reperfusion (Fig 1). However, TUNEL staining was still undetectable on the contralateral hemisphere that was not affected by MCAO after 24 h of reperfusion (Fig. 2a), but was abundant in both the core region and the cortical areas around the infarct (Figs. 1c & 2b & c). Further, TUNEL positive cells formed a sharp line of demarcation across the lateral ventricle, which separates the ischemic and the fully perfused brain areas (Fig. 2d).

In parallel, we also observed a marked induction of the mitotic proteins, cyclin B1 and MPM-2, in the ischemic area, which reached a peak in intensity at 24hr of reperfusion. In the hemisphere contralateral to the occlusion, these mitotic epitopes were observed only sporadically, and most positive cells had the morphology of endovascular epithelial cells (Fig. 2a). However on the ischemic side, these mitosis positive cells were widespread and intensely stained, with the highest density of immunoreactivity observed in the frontoparietal cortex (Fig. 2b & c). Most of the immunoreactivity of cyclin B1 and MPM-2 was observed in the cytoplasm (Fig. 2c) with a few instances of nuclear localization. This cytosolic distribution of cdc2/cyclin B1 is consistent with the previous observation in AD patients(Husseman et al., 2000).

The regional staining pattern of TUNEL and cyclin B1/MPM-2 showed a high degree of colocalization. The staining was sporadic and very weak on the contralateral hemisphere and was predominantly confined to endothelial cells, but was intense in the ischemic regions and formed a sharp contrast across the lateral ventricle. Cyclin B1 (data not shown) or MPM-2 (Fig.2d) colocalized with TUNEL in about 30-70 percent of

neurons, depending on the brain location and severity of ischemic damage. Most of the cyclin B1 and MPM-2 positive cells in the frontoparietal cortex had a neuronal morphology (Fig. 2c).

Transient cerebral ischemia-reperfusion induces NFT immunoreactivity

We further assessed ischemic brain sections of animals that had undergone MCAO with a variety of antibodies that detect neurofibrillary tangles in neurons affected with AD. These monoclonal antibodies are directed against distinct phosphorylation sites and/or conformational states of tau protein present in AD brains (Goedert et al., 1994). We observed positive staining with 5 different NFT specific antibodies (Fig. 3a, c-f). The NFTs were expressed throughout the ischemic area, with the most intense immunoreactivity in the frontoparietal cortex (Fig. 3a, c-f), colocalized with the TUNEL/cyclin B1/MPM-2 staining. Although there was evidence of cell shrinkage and nuclear condensation, these NFT positive cells still had an apparent neuronal morphology, and most of the immunoreactivity was localized in the cytoplasm (Fig. 3). In addition, the AD-related neuropathological marker, APP phosphorylated on Thr 668 (Caporaso et al., 1992), was also observed in neurons in the ischemic area (Fig. 3g).

Immunoblot analysis of cortical and basal ganglion extracts revealed a marked induction of NFT specific epitopes in cortical areas affected by MCAO-induced ischemia (Fig. 3h), compared with the control areas of the contralateral cortex. However, defined tangles and senile plaques, as identified with modified Gallyas silver and thioflavin-S staining could not be detected, except for a number of silver-stain positive cells. Similar

assessments in rats at 30 days after the ischemic event were also negative for Gallyas silver or thioflavin-S staining.

Previous studies have demonstrated that tau can be phosphorylated by a number of protein kinases (Pelech, 1995). We examined several candidate protein kinases using immunoblotting. We observed an increase in cdc2 protein kinase levels (Fig. 4a), as well as GSK3-β and pERK levels (Fig. 4b) in cortical extracts from animals at 24 h after the ischemic event. Theses increases in protein levels were not significantly different when compared to control expression levels in the contralateral brain hemisphere. However, consistent with our immunohistochemical results (Fig 2), a marked induction of cyclin B1 was observed using immunoblotting (Fig. 4b).

Colocalization of markers of apoptosis, mitosis and NFTs

Markers of apoptosis, mitosis and NFTs were highly colocalized in neurons. Double-label staining indicated a colocalization in 30-70% in TUNEL positive cells of NFTs in the frontoparietal cortex (Fig 5a). Cyclin B1 was colocalized with cdc-2 (Fig 5b) and the neuronal marker, MAP2B (Fig. 5c), and NFTs (Fig. 5c).

17-β estradiol reduces ischemic damage, aberrant mitosis and NFT epitopes.

To determine the correlation between ischemic damage and NFT formation, we assessed the response to MCAO in the presence of a known anti-apoptotic agent. Estrogen treatments have been demonstrated to be effective in protecting greater than 50% of ischemic brain tissues from MCAO-induced apoptosis (Simpkins et al., 1997). 2,3,5-Triphenyltetrazolium chloride (TTC) staining was used to reveal the extent of the

infarcted area after ischemia-reperfusion (Fig. 6a). 17 β-estradiol treatments in this stroke model reduced the ischemic volume by about 50% (Fig. 6a, b), and caused a similar reduction in TUNEL staining (Fig 6a, b) in the ischemic areas. We found that this treatment significantly reduced the expression of the mitotic protein, cyclin B1 (Fig. 6c), as well as of NFT epitopes in cortical brain extracts from the ischemic cerebral cortex (Fig. 6d).

Discussion

Herein we report for the first time, the early appearance of apoptosis and the subsequent induction of aberrant mitotic proteins and immunoreactivity associated with neurofibrillary tangles in a focal cerebral ischemia animal model for human strokes. Also, this is the first description of neurofibrillary tangles in a non-transgenic rodent model.

AD is characterized by pathological features that include senile plaques and neurofibrillary tangles (NFTs), the latter of which is composed of intracellular aggregates of hyper-phosphorylated tau protein. There remains controversy as to how these markers are pathogenically related and their role in the initiation and progression of neurodegeneration is not known (Vincent et al., 1996). Transgenic mice that host the familial disease mutations in APP and/or presenilins developed senile plaques, but lack NFTs and exhibit little neuronal loss (Goate et al., 1991; Sherrington et al., 1995; Holcomb et al., 1998). This suggests that the development of senile plaques and NFTs may have independent causative mechanisms during the pathogenesis of AD. NFT are a common feature of many neurodegenerative diseases, including Down syndrome, frontotemporal dementia linked to chromosome 17, and Parkinson-amyotrophic lateral sclerosis complex of Guam (Sanchez et al., 2001). As such, NFTs could initiate and/or

contribute to the progression of neurodegenerative diseases. Our results suggest that NFT formation may be a consequence of preceding ischemic events that results in the activation of mitotic proteins involved in the hyperphosphorylation of tau. In such a case, formation of NFTs may represent a downstream marker of earlier pathologies in neurons, so called "tombstones".

A consistent feature of tauopathy-related neurodegenerative diseases is the induction of aberrant mitotic proteins in affected neurons. The mitotic cdc2 kinase and its activator cyclin B1, as well as mitotic phosphoepitopes accumulate in degenerating neurons that contain the characteristic structural lesions of AD (Vincent et al., 1996; Husseman et al., 2000). The common appearance of mitotic proteins and NFTs in neurodegenerative diseases and their coincident appearance in cells undergoing apoptosis subsequent to an ischemic event indicate that apoptosis leads to activation of mitotic proteins which hyperphosphorylate tau.

Cell cycle signaling mechanisms are activated during apoptosis in neurons (Shirvan et al., 1997; Nuydens et al., 1998; Sakai et al., 1999) and insult-related induction of cell-cycle proteins has been studied in both *in vitro* and *in vivo* models (Nuydens et al., 1998)(Lin et al., 2001). In post-mitotic neurons of the adult brain, active mitotic kinase complexes induce an abortive or a catastrophic M-phase and eventually lead to cell death. Ischemic neuronal death may involve cell-cycle signaling, as cyclin D1, cyclin G1, PCNA or p21 waf1/cip1 are induced (Tomasevic et al., 1998; van Lookeren Campagne and Gill, 1998) in vulnerable neurons following transient global ischemia. However, in neurons, there is no evidence, such as DNA replication, for normal progression in mitosis.

The activation of cdc2 in the neurons may be fundamental to the progression to neuronal death and to the expression of NFT pathology (Vincent et al., 1996; Husseman et al., 2000). Tau protein is a high affinity substrate of cdc2 (Paudel, 1997). In differentiated neurons, cdc2-induced phosphorylation of tau inhibits its binding to tubulin, and reduces the stability of the cytoskeleton (Lu et al., 1999). Nucleolin, another cdc2 substrate, is crucial for ribosomal biogenesis and cytoplasmic-nuclear shuttling of RNA, and it may contribute to AD pathology (Dranovsky et al., 2001). During mitosis in dividing cells, phosphorylation of these cdc2 substrates is necessary for dynamic microtubule reorganization, and the reduction of transcriptional activity. However in differentiated neurons, this mitotic protein activation may contribute to cell death signaling and initiate the formation of neurofibrillary tangles. Indeed, DNA degradation and caspase activation are increased in both AD and other degenerative disorders, and suggested to play a key role in the pathogenesis of these diseases (Marx, 2001).

Estrogens are potent anti-apoptotic agents against a variety of insults (Green and Simpkins, 2000). Our ability to prevent apoptosis and reduce mitotic proteins and NFTs with an estradiol pretreatment suggests that prevention of the generation of signaling components upstream of apoptosis can also prevent or diminish subsequent mitotic and tau hyperphosphorylation responses to an ischemic event. Since initiation of apoptosis is an early event that eventually leads to tau pathology, anti-apoptosis therapeutic strategies are reasonable approaches to the prevention of NFT formation. By inference, therapies aimed at prevention of NFTs, without treating apoptosis and cell cycle protein activation may not be effective.

Finally, the present data also suggest that sporadic hypo-perfusion of the brain may initiate and contribute to the progression of neuropathology in non-familial AD patients, and may provide the mechanisms of the long-observed correlation between the ischemic events and the increased prevalence of AD. As such, prevention of ischemic episodes may serve as an additional therapeutic approach to prevent the initiation and progression of AD.

Acknowledgement: We thank Dr. Peter Davis, Albert Einstein College of Medicine, for the NFT specific monoclonal antibodies. This study was supported by the grant from NIH (A610485) and MitoKor.

Methods:

Animal surgeries: For middle cerebral artery occlusion and reperfusion, an intraluminal filament model was used (Bremer et al., 1975). All surgeries were performed at 14 day after ovariectomy. To achieve a transient MCAO, the internal carotid artery (ICA) was exposed, and a 3-0 monofilament nylon suture was introduced into the ICA lumen through a puncture and was gently advanced to the distal internal carotid artery (ICA) until proper resistance was felt. After 1 h, the suture was withdrawn from the ICA and the distal ICA was immediately cauterized, each treatment or surgery includes at least 3-4 animals as a group.

Estrogen treatments: 17β-Estradiol (Steraloids, Inc. Newport, Rhode Island) was dissolved in absolute ethanol and then corn oil (Penta Manufacturing airfield, New Jersey) at a concentration of 100μg/ml. Ethanol was evaporated by incubation at 60°C over night. A single subcutaneous injection of 100 μg/kg, or vehicle was given to rats at 2 hours before MCAO surgery.

Immunohistochemical staining: For pathological studies, we used perfusion/immersion fixed (4% paraformaldehyde) paraffin-embedded brain sections (5 to 7 mm thick) that were analyzed with the following methods. All single antigen detection was performed using an avidin-biotin immune-peroxidase method with DAB substrate (dark brown) and Histostain plus kits (Zymed, Inc, South San Francisco, CA) according to the manufacturer's instruction. Some of the stains were counter-stained with heamatoxylin (blue) for better microscopy resolution.

Multi-antigen immuno-detection or colocalizations were achieved either with immunofluorescence with a variety of highly species-specific or isotype-specific non-overlapping Alexa-Fluorochrome conjugated secondary antibodies (Molecular Probes, Inc. Eugene, OR), or with different enzyme-substrate system (peroxidase VS alkaline phosphotase) with double stain kit (Zymed, Inc, South San Francisco, CA). Imuunofluorescence stainings were counter-stained with DAPI, a nuclear marker.

TUNEL assay and colocalizations with specific antigens: DNA fragmentation was detected by a TUNEL method using Dead END fluorescent kit (Promega, Madison, WI), according to the instruction of the manufacture. Colocalization with TUNEL staining was performed using an immunofluorescence method. Specific antigen was detected by highly specific Alexa-Fluorochrome conjugated secondary antibodies that does not overlap with FITC labeled TUNEL stain (Molecular Probes, Inc. Eugene, OR).

Antibodies used in the assays include MAP-2B (1:50) (Sigma. Louis, MO); Cyclin B1 (1:50), Cdc2 (1:50) (Santa Cruz. Santa Cruz, CA); MPM-2 (DAKO, Carpinteria, CA); Phospho-APP (Thr668) (Cell Signaling Technology, Inc. Beverly, MA).

The following antibodies were used to stain NFTs in brain sections. Conformational tau epitopes: TG3 (Phospho-tau 231/235 and AD conformation); Phosphorylated tau epitopes: CP13 (Phospho-tau 202/205), CP3 (Phospho-214), PHF-1 (Phospho-tau 396/404), and CP9 (Phospho-tau 231) at 1:10 dilution. All of the above mentioned NFT specific-antibodies were characterized by and are the kind gift of Dr. Peter-Davies (Albert Einstein College of Medicine).

Immunoblotting: For immunoblotting analysis with various antibodies, brain tissue was dissected into frontoparietal cortex and subcortical basal ganglia fractions, and homogenized in lysis buffer (containing protease and phosphatase inhibitors). Samples were centrifuged at 12,000g for 30 min, and supernatants were collected for analysis. Samples were probed with specific antibodies at proper dilutions with normal procedures. The blots were developed with an enhanced chemiluminescent kit (Pierce, Rockford, IL). In all immunoblots, each lane represented an independent experimental animal with described treatments.

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Figure Legends:

Figure 1: Early induction of apoptosis and mitotic proteins following ischemia-reperfusion.

Apoptosis (TUNEL staining) and mitotic proteins (cyclin B1 and MPM-2) are induced at different time intervals after ischemia-reperfusion. (a) As early as 2h of reperfusion, TUNEL was detected in the basal ganglion, the core region of the infarct, but cyclin B1 and MPM2 staining neurons were not observed. (b) At 4h of reperfusion TUNEL staining was more intense, and extended to the frontoparietal cortex; cyclin B1 and MPM2 immunoreactivity was observable in the core region. (c) At 24 h, there was extensive TUNEL, cyclin B1 and MPM-2 staining throughout the core and frontoparietal cortex. (d) TUNEL, cyclin B1, and MPM2 positive neurons at 4h reperfusion in the frontoparietal cortex. All photomicrographs were taken from adjacent brain slices in representative experiments. Scale bar = 100μm.

Figure 2: Extensive expression of apoptotic and mitotic markers at 24h of reperfusion.

At 24 hr of reperfusion, apoptosis, as well as mitotic proteins were evident throughout the infarct. (a) TUNEL as well as cyclin B1 and MPM2 staining was negative in the contralateral cortex. (b-c) TUNEL and cyclin B1/MPM-2 staining was seen throughout the infarct, including the core (b), and the peri-infarct cortex. (c) TUNEL (left), cyclin B1 (center) and MPM-2 (right) in the cortex. (d) Immunofluorescence of DAPI, a nuclear counterstain (blue), TUNEL (green), MPM-2 (red) and the merged

image near the ipsilateral lateral ventricle, which separated the ischemic from the fully perfused area. Scale bar = 100μm.

Figure 3: Induction of neurofibrillary tangle immunoreactivity in ischemic areas

Immuno-peroxidase staining of TG3 in the ipsilateral (a) and contralateral (b) frontoparietal cortex. The upper left frame shows the staining at 40 X magnifications. Depicted are immunoperoxidase stainings of PHF-1 (c); CP-3 (d); CP-9 (e); CP-13 (f); p-APP (g), respectively. In a-f, haematoxylin nuclear counter-stain appears as blue and positive DAB immunostaining as dark brown signals. (h) Immunoblotting of NFT specific epitopes in the contralateral cortex (lane 1-4) and ischemic frontoparietal cortex (lane 5-8). Each lane in h represents an individual experimental animal. Scale bar = 100μm.

Figure 4: Immunoblotting of protein kinases functionally relevant to tau hyperphosphorylation.

(a) Immunoblotting of cdc2/P34 protein kinase. Lanes 1-3 were from the contralateral cortex and lanes 4-6 were from the ischemic cortex. (b) Immunoblotting of cdk5; GSK3-β; p-ERK; cyclin B1; and β actin as a loading control. In b Lanes 1-4 were from the contralateral cortex and lanes 5-8 were from the ischemic cortex. Each lane represents a sample from an independent experimental animal.

Figure 5: Colocalization of markers of apoptosis, mitosis and NFTs

Colocalizations of multiple antigens detected by immunofuorescence (a-b) and chromagenic staining (c-d). (a) PHF-1(red) colocalizes with TUNEL (green); (b) cyclin B1 (red) colocalizes with cdc2 kinase (green). (c) cyclin B1 (red) colocalizes with MAP2 (dark blue); (d) cyclin B1(red) with PHF-1 (dark blue). Scale bar = 100μm.

Figure 6: Effects of 17 β-E2 treatment on the NFT phospho-epitope and cyclin B1.

Typical TTC and TUNEL stained brain slices from ovariectomized (a) or 17 β-E2 treated ovariectomized rats (b) at 24 h following MCAO. The circle indicates the cortical area from which the TUNEL stained slices were obtained. (c) Immunoblot analysis of cyclin B1 of the brain extracts from ovariectomized contralateral cortex (lane 1-3), 17 β-E2 treated ipsilateral cortex (lane 4-6) and ovariectomized ipsilateral cortex (lane-7-9) with β-actin as a loading control (lower panel). (d) Immunoblot analysis of NFT epitopes in ovariectomized rat ipsilateral cortex (lane 1-3) and 17 β-E2 treated ipsilateral cortex (lane 4-6). Each lane represents a sample from individual experimental animals. Scale bar = 100μm.

Fig. 1

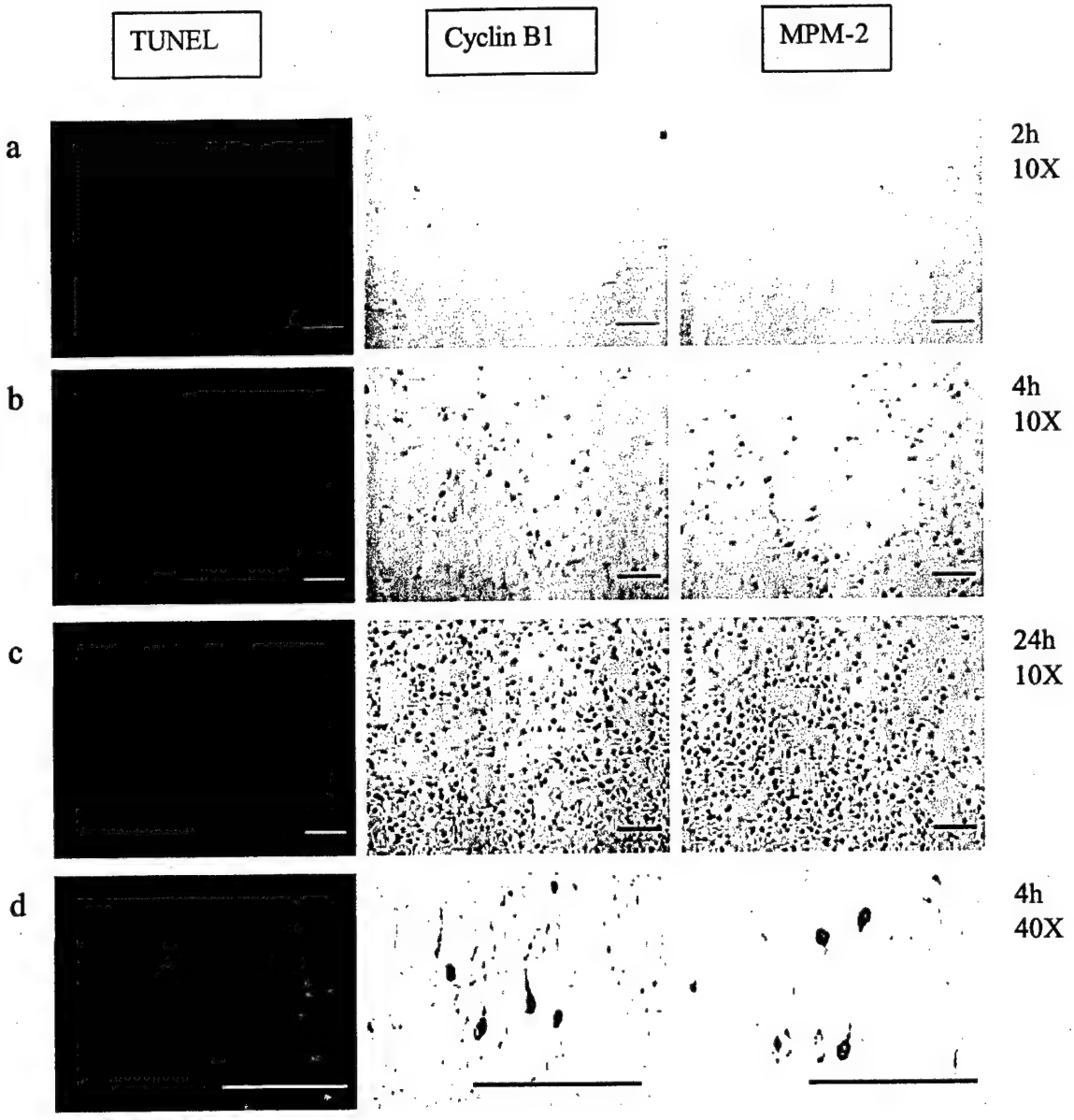
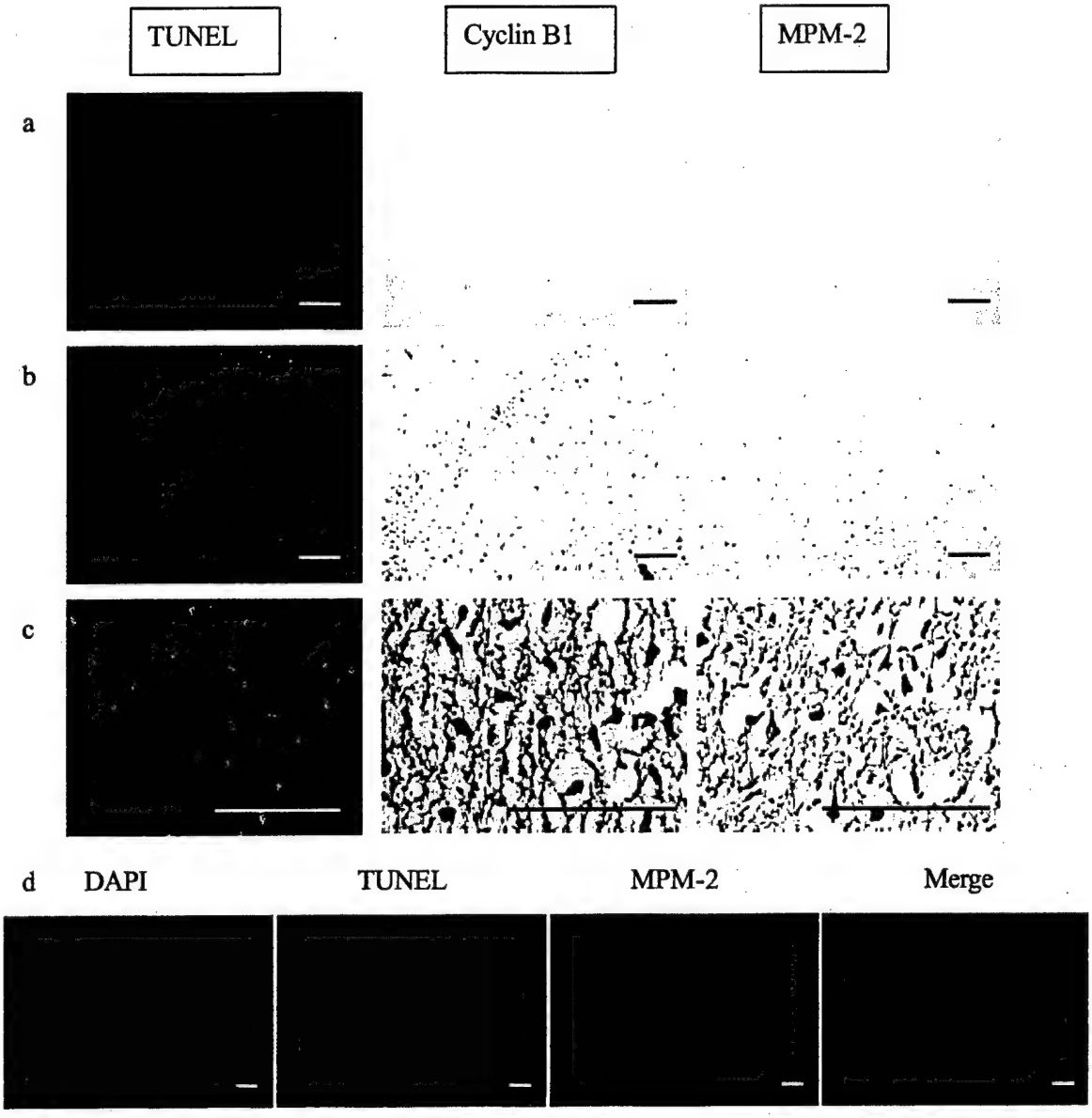


Fig. 2



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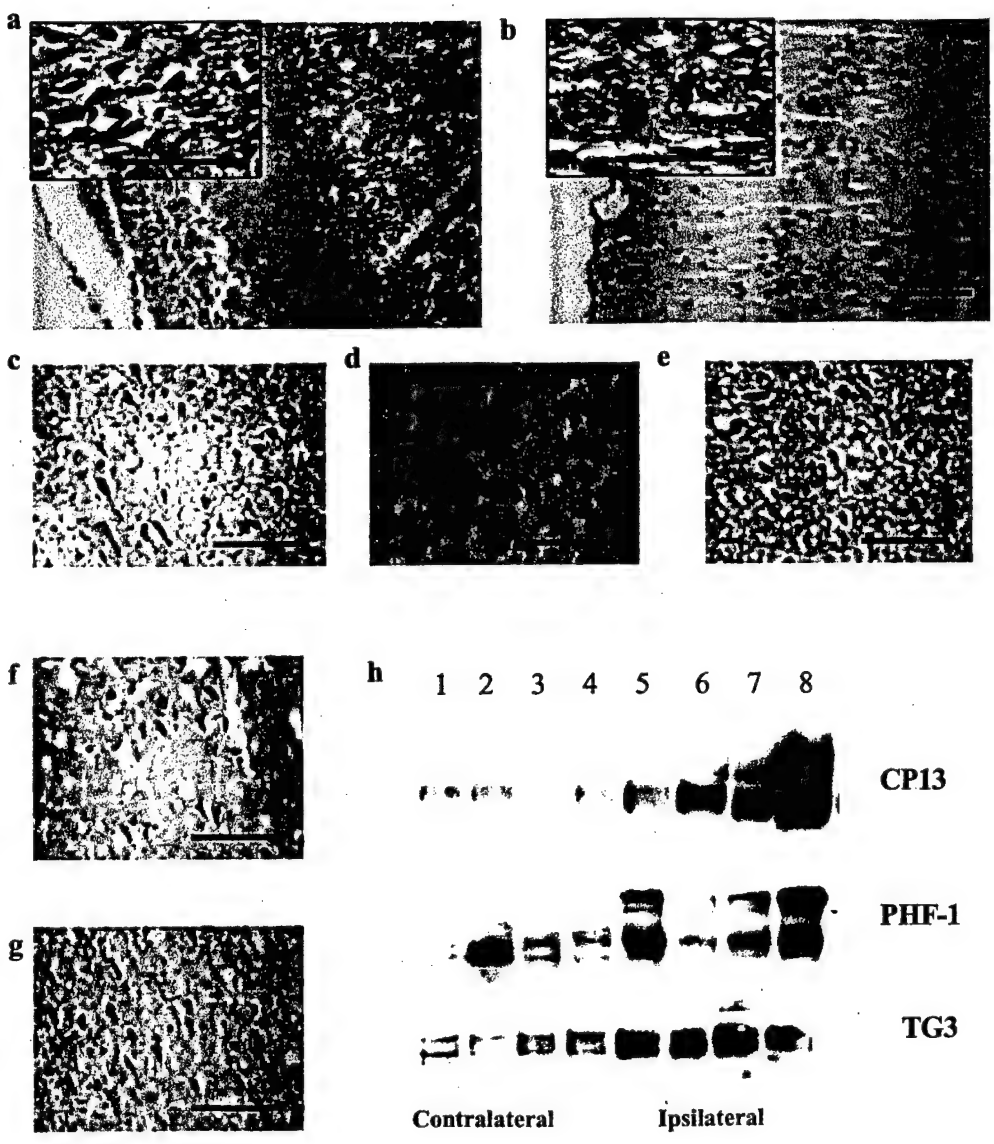
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Fig. 4

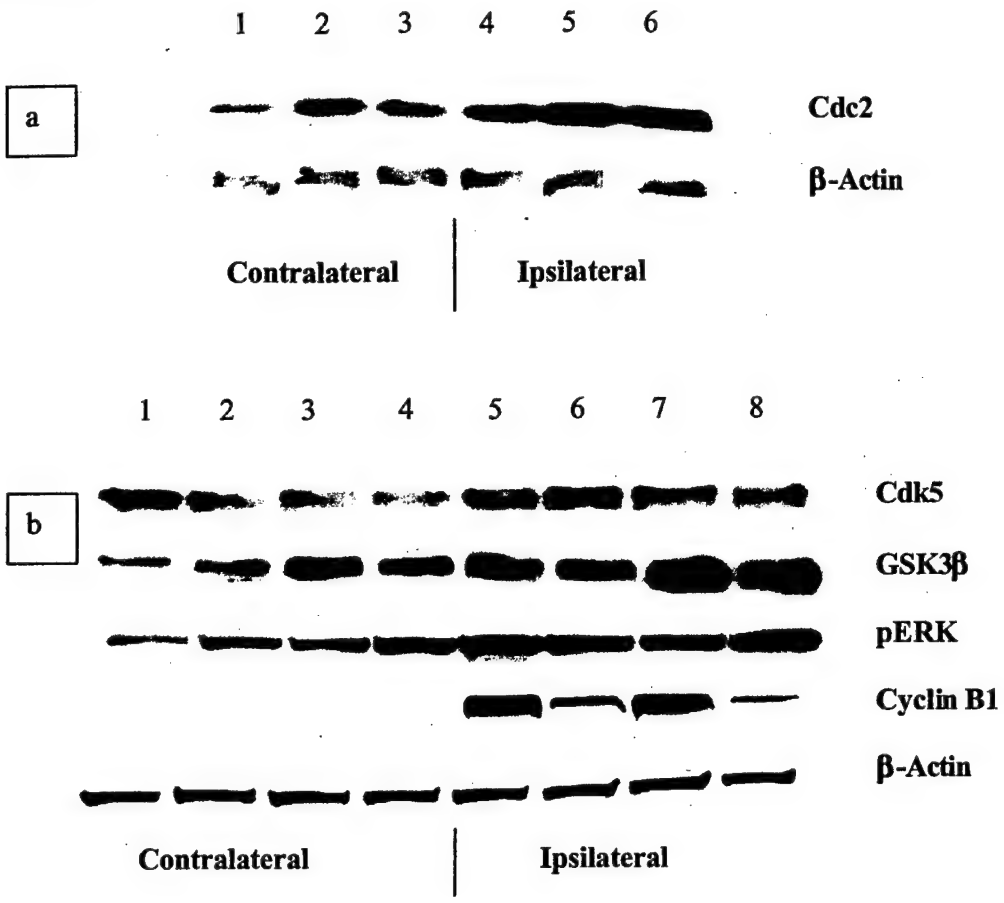
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Fig. 5

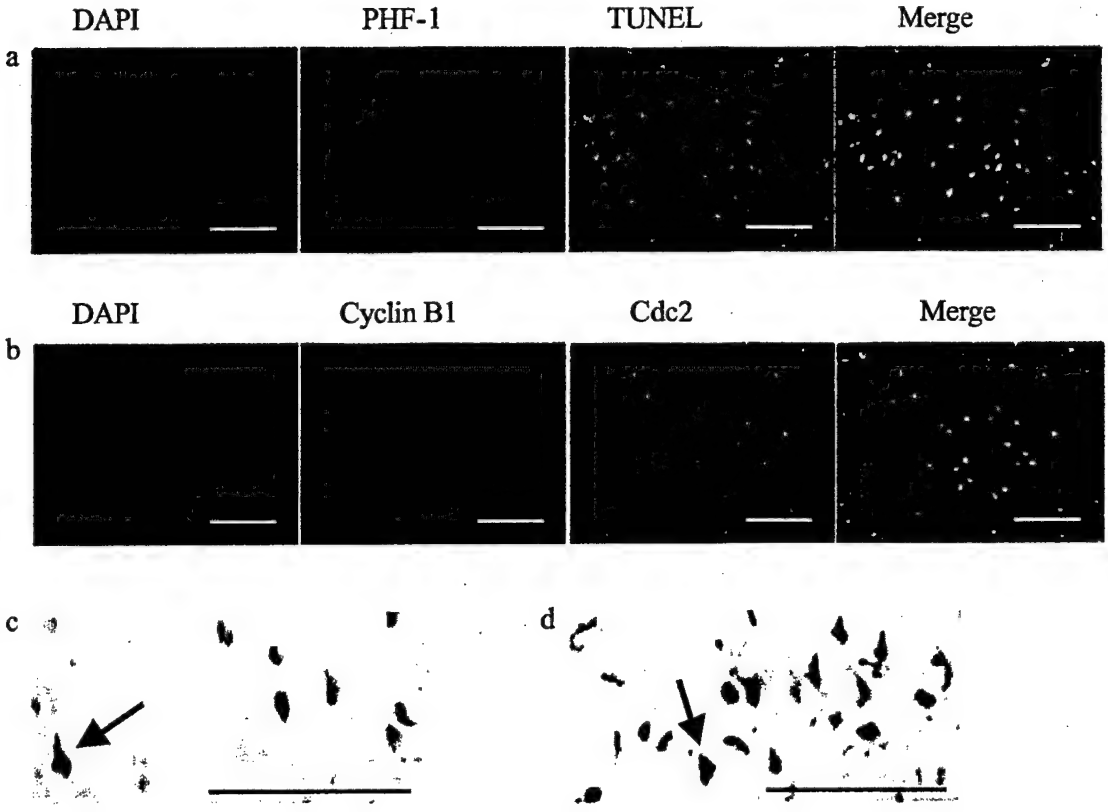
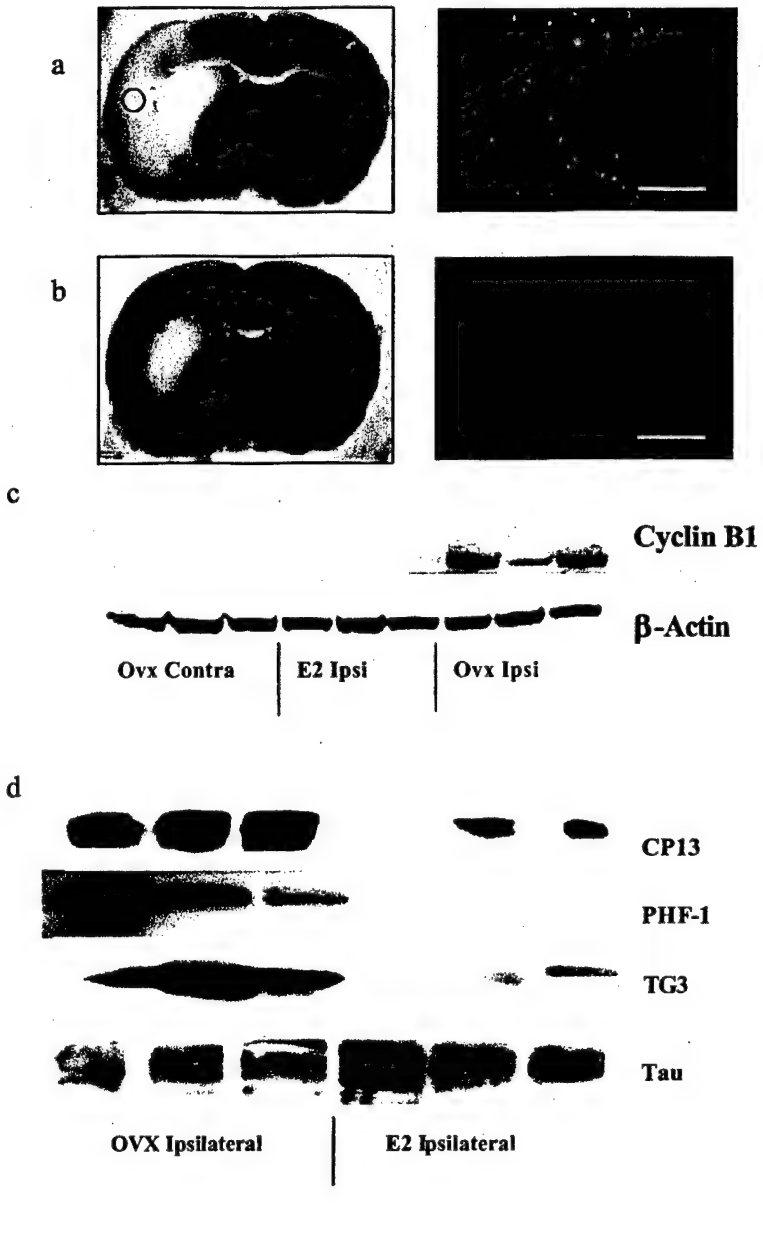


Fig. 6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Appendix O

Estradiol protects against ATP depletion, mitochondrial membrane potential decline and the generation of reactive oxygen species induced by 3-nitroproprionic acid in SK-N-SH human neuroblastoma cells

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Abstract

Mitochondria are recognized as modulators of neuronal viability during ischemia, hypoxia and toxic chemical exposure, wherein mitochondria dysfunction leading to ATP depletion may be a common pathway of cell death. Estrogens have been reported to be neuroprotective and proposed to play a role in the modulation of cerebral energy/glucose metabolism. To address the involvement of 17β-estradiol preservation of mitochondrial function, we examined various markers of mitochondrial activity in human SK-N-SH neuroblastoma cells exposed to 3-nitroproprionic acid (3-NPA), a succinate dehydrogenase inhibitor which uncouples oxidative phosphorylation. 3-NPA (10 mM) significantly increased ATP levels at 2 h then caused a 40% and a 50% decrease in ATP levels from baseline when treated for 12 h and 24 h, respectively. 3-NPA also induced significant increases in levels of cellular hydrogen peroxide and peroxynitrite at 2 h and a 60% decrease in mitochondrial membrane potential (MMP) at 12 h exposure. 17β-Estradiol (17β-E_2) pretreatment restored the ATP level back to 80% at 12 h of that in control cells treated with 3-NPA but without E_2, blunted the effect of 3-NPA on MMP and reactive oxygen species levels. The present study indicates that 17β-E_2 can preserve mitochondrial function in the face of inhibition of oxidative phosphorylation.

Keywords: ATP depletion, 17β-estradiol, mitochondrial membrane potential, 3-nitroproprionic acid, reactive oxygen species.

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Mitochondria are unique organelles in their involvement in the consumption of oxygen, production of ATP, oxygen radicals, and mobilization of calcium (Gunter and Pfeiffer 1990; Melov 2000). Mitochondria play pivotal roles in controlling cell death by mechanisms including: (i) disruption of electron transport, oxidative phosphorylation and ATP production; (ii) release of caspase-activating proteins (cytochrome c, Apaf-1, procaspase-9, etc.); and (iii) generation of reactive oxygen species and alteration of cellular redox potential (Green and Reed 1998). Increasing evidence shows that mitochondrial dysfunction is involved in neuronal damage associated with ischemia, hypoxia, toxicant exposure, and neurodegenerative diseases (Lemasters *et al.* 1997; Beal 1998).

Estrogens have long been recognized as antioxidants and recent studies have showed that estrogens are also potent neuroprotective agents. *In vitro* studies revealed that estrogens reduced the cellular toxicity of beta-amyloid peptide (Aβ) and other oxidative insults (Behl *et al.* 1995; Goodman *et al.* 1996; Green *et al.* 1997) and this correlated with a decrease in production of lipid peroxides (Lacort *et al.* 1995; Miura *et al.* 1996). 17β-Estradiol (17β-E_2) protected neurons from glutamate toxicity (Keller *et al.* 1997; Singer *et al.* 1999). *In vivo*, treatment with 17β-E_2 or 17α-E_2

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Abbreviations used: DCF-DA, 2,7-dichlorofluorescein diacetate; DHR123, dihydrorhodamine 123; 17β-E_2, 17β-estradiol; FBS, fetal bovine serum; G3PD, glyceraldehyde-3-phosphate dehydrogenase; GSH, glutathione; MMP, mitochondrial membrane potential; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 3-NPA, 3-nitroproprionic acid; PBS, phosphate-buffered saline; Rh123, rhodamine 123; SDH, succinate dehydrogenase.

markedly reduced ischemic brain damage induced by middle cerebral artery occlusion in ovariectomized rats (Simpkins *et al.* 1997; Yang *et al.* 2000) and estrogens protected against vulnerability of the lateral striatal artery to 3-NPA (Nishino *et al.* 1998).

Several studies have shown that estrogens may exert direct or indirect effect on mitochondrial function. Mitochondrial genes were potential sites of primary action of steroid hormones (Demonacos *et al.* 1996). 17β-E_2 stabilized mitochondrial function against actions of mutant presenilin-1 (Mattson *et al.* 1997) and inhibited mitochondrial F0F1-ATP synthase/ATPase by binding to one of its subunits (Zheng and Ramirez 1999a, b)

To address the role of mitochondria in the neuroprotection effects of estrogens, we chose 3-nitroproprionic acid (3-NPA), a succinate dehydrogenase inhibitor, to model condition in which interrupted energy metabolism is observed, such as cerebral ischemia (Hansford 1985; Du *et al.* 1996; Fink *et al.* 1996) and chronic neurodegenerative disease (Beal 1995). We assessed the ability of 17β-E_2 to affect the 3-NPA modulation of ATP production, mitochondrial membrane potential, reactive oxygen species and cell viability.

Materials and methods

Materials

SK-N-SH cells were obtained from American Type Tissue Collection (Rockville, MD, USA), RPMI-1640 media from GibcoBRL (Grand Island, NY, USA), 3-nitroproprionic acid, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma Chemical Co. (St Louis, MO, USA). ATP determination kit, rhodamine-123 (Rh123), 2,7-dichlorofluorescin diacetate (DCF-DA), dihydrorhodamine 123 (DHR123), and Hoechst 33258 were purchased from Molecular Probes (Eugene, OR, USA). 17β-E_2 was from Steraloids (Wilton, NH, USA).

SK-N-SH neuroblastoma cell culture

SK-N-SH cell cultures were maintained in RPMI-1640 media supplemented with 10% charcoal-stripped fetal bovine serum (FBS) in monolayers in plastic Corning 150-cm^2 flasks at 37°C and under 5% CO_2−95% air. Media was changed every 2 days. Cells were observed with a phase-contrast microscope (Nikon Diaphot-300, Nikon, Tokyo, Japan). SK-N-SH cells used in the following experiments were in passes 37–45.

MTT reduction

The effects of 3-NPA on mitochondrial succinate dehydrogenase (SDH) activity were assessed by indirectly measuring the conversion of the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan crystals. Cells were plated 2×10^4 cells per well in Nunc 96-well plate, and allowed growth in regular media for 48 h. Cell cultures were exposed to various doses of 3-NPA for 2–48 h and then were rinsed with media and incubate with 10 μL MTT (0.5 mg/mL). Following overnight solubilization of the formazan crystals in 50% *N,N*-dimethylformamide, 20% sodium dodecyl sulfate, pH 4.8, the optical density was determined at 575/690 nm.

Measurements of ATP levels

Experiments were initiated by plating 1×10^6 cells per well in 24-well plates, and allowing growth in regular media for 48 h. Cells were exposed to (i) various doses of 3-NPA for 2 h to 24 h. (ii) 10 mM 3-NPA for 2 or 12 h with or without various doses of 17β-E_2 (6 h pretreatment, continued through 2 or 12 h, respectively). Cellular ATP levels were quantified using a luciferin and luciferase-based assay. Cells were rinsed with phosphate-buffered saline (PBS) and lysed with ATP-releasing buffer containing 100 mM potassium phosphate buffer at pH 7.8, 2 mM EDTA, 1 mM dithiothreitol (DTT) and 1% Triton X-100; 10 μL of the lysate was taken for protein determination. Another 10 μL of the lysate was taken and added to Nunc 96-well plate. ATP concentrations in lysates were quantified using ATP determination kit according to the manufacturer's instruction. The 96-well plates were read using MLX Microtiter Plate Luminometer (Dynex Technologies Inc., Chantilly, VA, USA). A standard curve was generated using solutions of known ATP concentrations. ATP levels were calculated as nM of ATP per mg of protein and normalized to levels in untreated control cultures.

Assessments of mitochondrial membrane potential

We used the cationic and lipophilic dye Rh123 that permeates into the negatively charge mitochondria and therefore reflects the mitochondrial membrane potential (Johnson *et al.* 1980; Emaus *et al.* 1986) to examine the effects of 3-NPA on mitochondrial potentials. Cells were plated 1×10^7 per 100 mm dish, allowing growth until confluent. Cell cultures were incubated for 12 h in the presence or absence of 3-NPA with or without 17β-E_2 (6 h pretreatment continued through 12 h) in an atmosphere of 95% air−5% CO_2. The mitochondria were stained by adding to cell cultures a stock solution of Rh123 (10 mM in methanol), to a final concentration of 1 μM and incubating for 15 min in the dark at room temperature. Cell cultures were then rinsed three times with the appropriate treatment medium followed by a 5-min wash with a HEPES-buffered control salt solution containing: 145 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, pH-adjusted to 7.4 with NaOH. Cells were suspended and centrifuged at 300 *g* for 2 min and resuspended in the salt solution at a concentration of 1×10^6 cells/mL. The cell suspensions were then filtered through a nylon mesh screen of 40 μm into borosilicate glass tubes at a cell density of 1 mL of the cell suspension (10^6 cells) per tube. Changes in Rh123 fluorescence intensity were measured by FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) with excitation at 488 nm using an air-cooled argon-ion laser and emission at 525 nm using an optical band-pass filter.

Measurement of reactive oxygen species

Levels of cellular oxidative stress were estimated by monitoring levels of hydrogen peroxide and peroxynitrites. Cells were plated 1×10^7 per 100 mm dish, allowing growth until confluent. Cells were then exposed to 10 mM 3-NPA or 17β-E_2 for 2 h, or 10 mM 3-NPA with 17β-E_2 6 h pretreatment (continued through 2 h). Following the indicated treatment, cells were stained with either 2,7-dichlorofluorescin diacetate (DCF-DA) or dihydrorhodamine 123 (DHR123). Cells were incubated with 40 μM DCF-DA or

The significant treatment effects on cell viability, MTT reduction, mitochondrial membrane potential, reactive oxygen species were determined using ANOVA followed by Tukey's *post hoc* test, with significance determined at $p < 0.05$.

Results

Effects of 3-NPA on conversion of MTT to formazen

3-NPA caused time- and dose-dependent decline in formazan production as measured by the inhibition of reduction of MTT to formazan crystals (Fig. 1a). When measured at 24 h, the 10 mM concentration of 3-NPA caused an approximate 50% reduction in formazen formation (Fig. 1a).

Effects of 3-NPA, 17 β-E_2 and their combination on ATP concentrations

In control cultures, cellular ATP concentrations were 27.8 ± 7.0 nmol/mg protein. 3-NPA caused time- and dose-dependent changes in ATP levels (Fig. 1b). Exposure to 3-NPA at concentrations ranging from 1 mM to 20 mM resulted in a significant increase in ATP levels at 2 h. The concentrations of 5–20 mM 3-NPA also caused a time-related decrease in ATP levels at 4 h and thereafter exposures. The elevations of ATP levels in 1 mM 3-NPA-treated cells after 4 h were not statistically significant (Fig. 1b). At 12 h, the 10 mM, 15 mM and 20 mM concentrations of 3-NPA-induced declines in ATP of approximately 40%, 60%, and 80%, respectively, from control (0 h) values (Fig. 1b). ATP level had further decrease at 24 h to about 50%, 70%, and 95% of that of control cells (Fig. 1b).

We then evaluated the effect of 17β-E_2 on the ATP changes induced by 2 and 12 h of exposure to 3-NPA. Treatment with 17β-E_2 alone for 12 h exerted no significant effects on cellular ATP levels (data not shown). Pretreatment with 17β-E_2 caused a dose-dependent blockade of the increase in cellular ATP induced by 3-NPA (Fig. 2). Similarly, 17β-E_2 pretreatment blunted the decline in

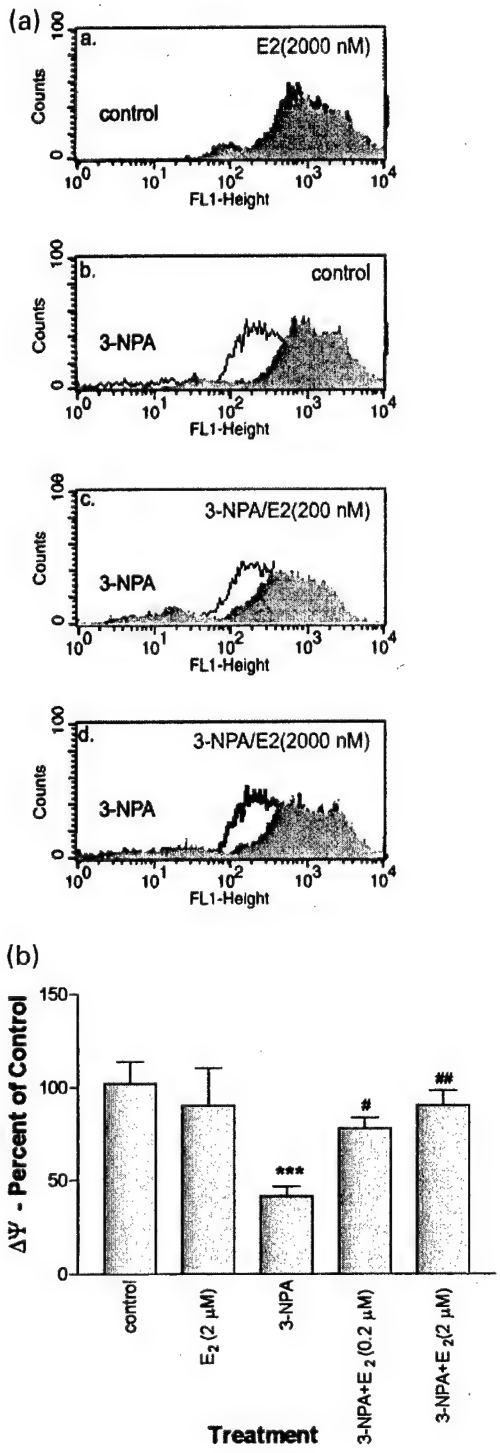

Fig. 4 Effect of 6 h pretreatment with 17β-E_2 on 3-NPA induced changes in mitochondrial membrane potentials. (A) Analysis of rhodamine 123 fluorescence in SK-N-SH cells by FACScan. Cells were positively stained with rhodamine 123. Increasing fluorescence intensities were plotted on the *x*-axis in log fluorescence units vs. cell number on the *y*-axis. The FL-1 'high' population is displayed in gray on the histograms and the FL-1 'low' population is represented by the black overlay. (a) Cells treated with 2 μM 17β-E_2 (gray) and control cells (black). (b) Control cells (gray) and cells treated with 10 mM 3-NPA for 12 h (black). (c) Cells exposed to 10 mM 3-NPA for 12 h with 6 h 17β-E_2 (0.2 μM) pretreatment (gray) and cells only exposed to 10 mM 3-NPA for 12 h (black). (d) Cells exposed to 10 mM 3-NPA for 12 h with 6 h 17β-E_2 (2 μM) pretreatment (gray) and cells only exposed to 10 mM 3-NPA for 12 h (black). (B) Quantitative summary of rhodamine 123 fluorescence studies, comparing changes in relative fluorescence intensities under indicated treatments. Values are expressed as a percentage of control levels and represent the mean ± SEM for 4–8 cultures/group. The control value was 159 ± 7 fluorescence units. #$p < 0.05$ compared with the 3-NPA group. ##$p < 0.01$ compared with the 3-NPA group. ***$p < 0.001$ compared with the control or 17β-E_2 group.

attenuated this response to 3-NPA (Fig. 6). Prolonged incubation (2–7 days) with lower dose (0.2 μM) similarly attenuates 3NPA-induced apoptosis (data not shown).

Discussion

The present study demonstrates that in cultured SK-N-SH human neuroblastoma cells, 3-NPA toxicity is associated with an early increase in ATP production and the generation of ROSs, followed by a reduction in ATP, a decline in mitochondrial membrane potential and subsequent apoptosis. Our observations that 17β-E_2 can attenuate each of these effects of 3-NPA suggest that the neuroprotective effects of this steroid may be related to its direct or indirect effects on mitochondrial function.

3-NPA is used both *in vitro* and *in vivo* to model acute insults such as ischemia (Geddes *et al.* 2000), as well as neurodegenerative disorders, including Huntington's (Beal 1994) and Alzheimer's disease (Beal 1995). 3-NPA-induced cell death has been proposed to result from the synergistic effects of inhibition of SDH and activation of glutamate receptors, which included rapid necrotic and delayed apoptotic cell death (Pang and Geddes 1997). The temporal pattern of cellular response to 3-NPA indicates that an elevation in ATP is associated with an increase in ROSs within 2 h of treatment. These changes are followed by a decline in ATP, a reduction in mitochondrial membrane potential by 12 h, and progression through apoptosis by 24 h. This pattern suggests that activation of mitochondrial respiration and production of ROSs are among the earliest effects of 3-NPA. Since increased calcium level in the mitochondria can initially stimulate mitochondrial respiration (Hansford 1985; McCormack *et al.* 1990), a primary neurotoxic action of 3-NPA may be Ca^{2+} dyshomeostasis in close association with increased production of ROSs, rather than an inhibition of SDH, which occurs later. This early increase in ATP production, however, is not necessarily associated with the later decline in ATP concentration and cell death, since an increase in ATP is observed at low doses of 3-NPA that do not result in the subsequent ATP loss or cell death. The late loss of ATP is likely associated with a cell death program as it has been reported that a substantial loss of ATP is a very late event in apoptosis in PC12 cells deprived of NGF (Mills *et al.* 1995).

3-Nitroproprionic acid provoked generation of reactive oxygen species, decline in mitochondrial membrane potentials and depletion of intracellular ATP which precede cell death. In cells with mitochondrial dysfunction, the glycolytic maintenance of ATP is important for generating ATP. The observed early 3-NPA-induced increase in the generation of ROSs can result in the loss of the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (G3PD) activity since acute exposure to 3-NPA reduces GSH level in rat brain (Binienda *et al.* 1998) and depletion of GSH can in turn inhibit G3PD by oxidation of critical thiol groups located at the active site of the enzyme (Brodie and Reed 1990; Vaidyanathan *et al.* 1993). Additionally, glutathione system is important for protecting neurons during impairment of energy metabolism (Zeevalk *et al.* 1998). Further, uncoupling oxidative phosphorylation could stimulate the F0F1-ATPase, causing hydrolysis of ATP generated by glycolysis and loss of glycolytic protection (Lemasters *et al.* 1997). Collectively, this indicates that multiple stresses induced by 3-NPA on the cells could directly or indirectly reduce available ATP.

We observed that estradiol pretreatment blunted or ablated each of the observed effects of 3-NPA, although the concentrations of the steroid needed to achieve the effects differed between parameters. The parameters most sensitive to estrogens were the time-dependent changes in ATP and the reduction in mitochondrial transmembrane potential induced by 3-NPA. Blockade of these effects of 3-NPA occurred at 17β-E_2 concentrations of 20–200 nM. Estradiol prevented the production of ROSs and apoptosis only at low μM concentrations. This marked difference in concentration of the steroid needed to antagonize the observed effects of 3-NPA could reflect the activation of different mechanisms at different concentrations of 17β-E_2 or may be related to the fact that the length of exposure to the steroid, magnitude of the stress and the timing of the observation can dramatically influence the dose-dependency of the effects of estrogens (Green and Simpkins 2000).

Pretreatment of 17β-E_2 prevented the mitochondrial depolarization in response to 3-NPA. This observation is consistent with the report that estrogens stabilized mitochondrial potential against actions of mutant presenilin-1 (Mattson *et al.* 1997). Whether the effect was due to the inhibition of the opening of permeability transition pores, the inhibition of the mitochondrial calcium uniport, or recovery of ATP levels, remains unknown. The maintenance by 17β-E_2 of mitochondrial membrane depolarization may be attributed to its antioxidant and/or ATP increasing effects.

Uncoupling oxidative phosphorylation could stimulate the F0F1-ATPase, causing hydrolysis of ATP. Recent studies show that estrogens can bind to one of the subunits of F1F0-ATPase (Ramirez *et al.* 1996; Zheng and Ramirez 1999a, b), and 17β-E_2 inhibited the F0F1-ATPase activity at micromolar concentration with 1–2 min of estrogen exposure (Zheng and Ramirez 1999b). Inhibition of F1F0-ATPase by 17β-E_2 could serve to blunt ATP loss.

The well characterized antioxidant activity of estrogens (Nakano *et al.* 1987; Sugioka *et al.* 1987; Hall *et al.* 1991) could account for both the maintenance of cellular ATP concentrations and mitochondrial transmembrane potential in the face of 3-NPA exposure. The antioxidant effects of 17β-E_2 could prevent lipid peroxidation in mitochondrial membranes (Lacort *et al.* 1995; Miura *et al.* 1996; Keller

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Appendix P

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Polycyclic phenols, estrogens and neuroprotection: a proposed mitochondrial mechanism☆

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Abstract

Polycyclic phenols, including the estrogens, have been shown to be potent neuroprotectants in a variety of cellular and animal model systems. Although classical estrogen receptor interactions and consequent responses play a role in certain circumstances, the neuroprotective activity of polycyclic phenols that do not interact with estrogen receptors ERα or ERβ is more likely to be through non-genomic mechanism(s). We propose here that such non-feminizing polycyclic phenols exert their protective effects at least in part by stabilizing mitochondria, preventing apoptotic and/or necrotic forms of cell death that are associated with mitochondrial dysfunction. Consistent with this mitochondrial model and the available data, these compounds protect neurons and other cell types from a wide variety of pathologically relevant stressors.

Keywords: Neuroprotection; Mitochondria; Estrogens; Steroids

1. Introduction

1.1. Neuroprotective activity of estrogens and polycyclic phenols

The neuroprotective effects of estrogens in neuronal cultures and primary cells have been widely described against a variety of toxicities including serum deprivation (Bishop and Simpkins, 1994; Green et al., 1997a), β-amyloid toxicity (Green et al., 1998; Pike, 1999) and oxidative stress (Behl et al., 1995; Goodman et al., 1996; Sawada et al., 1998, 2000), among others, in hippocampal, amygdala, cortical and mesencephalic neurons (recently reviewed by Green and Simpkins, 2000; Garcia-Segura et al., 2001; Lee and McEwen, 2001). Similarly, in rodent animal models, estrogens have been shown to attenuate neuronal loss following induction of cerebral ischemia in the middle cerebral artery occlusion model (Simpkins et al., 1997; Dubal et al., 1998; Yang et al., 2000), in vivo following kainic acid administration (Azcoitia et al., 1998), and in a contusion physical injury model (Nakamizo et al., 2000).

In many of the studies cited above with diverse types of cells and stressors, naturally occurring 17β-estradiol (17β-E2) and its inactive diastereomer, 17α- estradiol (17α-E2), have been found to be equally neuroprotective, despite the fact that 17β-E2 binds avidly to estrogen receptors α and β (ERs) and activates tissues in a hormonally-responsive manner, whereas 17α-E2 is biologically inactive at both receptors. These observations have led to a model in which neuroprotection can be achieved by a non-genomic mechanism not requiring action at the classical estrogen receptors. Further studies with a variety of similar compounds have confirmed that the structure-activity relationship for neuroprotection with this class of compounds differs significantly from the structural requirements for ER-dependent gene transcription (Behl et al., 1997; Green et al., 1997b; Moosman and Behl, 1999; Green et al., 2001). For example, the complete enantiomer of 17β-E2 (*ent*-17β-E2) has identical physiochemical properties as 17β-E2 except for interactions with other stereospecific molecules such as ERs. *Ent*-17β-E2 is reported to interact only weakly with uterine-derived ERs (Chernayaev et al.,

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1975; Payne et al., 1979) and lacks estrogenic effects on reproductive tissues in rodents (Terenius, 1968, 1971). In fact, some reports indicate that *ent*-17β-E2 exerts slight anti-uterotrophic activity and can antagonize the uterotrophic effects of 17β-E2 (Edgren and Jones, 1969; Terenius, 1971). We have reported that *ent*-17β-E2 exerts neuroprotective effects both in vitro and in vivo in the absence of stimulation of other estrogen-responsive tissues (Green et al., 2001). These studies and others have defined the minimal structural element required for neuroprotection to be the steroid A ring.

1.2. Role of mitochondria in cell death

Neurons, like other energy demanding tissues such as heart muscle, depend almost entirely on mitochondrial ATP production to meet their high energy demands and are at risk when ATP production is even transiently diminished. Damage to mitochondria leads to deficiency in ATP production, and to a concomitant increase in production of reactive oxygen species (ROS) that can overwhelm cellular antioxidant defense systems. Indeed, such mitochondrial dysfunction and failure are increasingly implicated as key events in the pathogenic cascade leading to ischaemic-mediated cell death from both necrosis and apoptosis (Lemasters et al., 1999; Kroemer and Reed, 2000). Under conditions of oxidative stress, especially when coupled with excessive cytoplasmic Ca^{2+} loading, mitochondria undergo a catastrophic loss of the impermeability of the inner mitochondrial membrane that causes a complete collapse of $\Delta\psi_m$, a process called permeability transition (PT). Such irreversible collapse is often accompanied by mitochondrial swelling and expulsion of cytochrome c into the cytoplasm, where it activates certain caspases and induces apoptotic cell death (Murphy et al., 1999; kroemer and Reed 2000). In normal tissues, various systems, including superoxide dismutase and catalase, moderate radical-induced damage by scavenging free radicals. Nonetheless, accelerated mitochondrial radical production can overwhelm such defenses, undermining cellular and mitochondrial integrity by inducing peroxidation of membrane lipids and impeding oxidative phosphorylation by inactivating Fe–S clusters that are parts of the electron transport system. The resulting acute loss of ATP causes the transmembrane ion-dependent ATPases to fail, thereby inducing necrosis from osmotic failure (Dykens, 1997, 1999).

In addition, mitochondrial failure initiates apoptosis in response to a variety of stressors, including decreased neurotrophic support, overexposure to excitatory amino acids such as glutamate, or injury. The excitotoxic process, for example, entails excessive stimulation of excitatory receptors, including NMDA and other voltage and metabotropic receptors, by exposure to elevated levels of glutamate. Excessive elevation of Ca^{2+} during glutamate excitotoxicity can undermine mitochondrial integrity and lead to acceleration of free radical production and ultimately to cell death (Dykens et al., 1987; Dykens, 1994). Mitochondria are thus believed to be key modulators of neuronal viability during conditions of excitotoxicity (Dykens, 1997). Therapeutic agents that are able to stabilize and augment mitochondrial function during conditions of ischemia are thus expected to be effective neuroprotectants, preventing apoptosis by maintaining functionally intact mitochondria.

Several studies have shown that estrogens and related compounds may exert direct or indirect effects on mitochondrial function. Zheng and Ramirez (1999a,b) reported that 17β-E2 can inhibit mitochondrial F0F1-ATPase by binding to one of its subunits. Other investigators have found that estradiol augments sequestration of cytosolic Ca^{2+} by mitochondria in the presence of glutamate (Nilson and Brinton, 2001). This may provide a neuroprotective role by removing cytotoxic calcium from the cytosol. In another study, 17β-E2 has been shown to stabilize mitochondrial function and thereby protect neural cells against the pro-apoptotic action of mutant presenilin-1 (Mattson et al., 1997). Anti-apoptotic action may also involve receptor mediated transactivation such as the up-regulation of Bcl-2, known to occur upon estradiol exposure.

To further illuminate the protective effects of estrogens as well as other polycyclic phenols on mitochondrial integrity, we examined a number of markers of mitochondrial activity in SK-N-SH neuroblastoma cells exposed to 3-nitroproprionic acid (3-NPA), which inhibits oxidative phosphorylation by blocking electron entry into electron transport at the level of succinate dehydrogenase (respiratory Complex II). This serves as a model of acute impaired energy production, such as occurs during cerebral ischemia (Hansford, 1985; Du et al., 1996; Fink et al., 1996), and also of more modest energy impairment associated with chronic neurodegenerative disease (Beal, 1998). We have previously reported that 17β-E2 prevents depletion of ATP, preserves membrane potential, and inhibits production of ROS caused by 3-NPA (Yang et al., 2000). Further studies reported here demonstrate that 17β-E2 as well as 17α-E2 increases the ability of mitochondria to retain their membrane potential, a marker of inner membrane impermeability and functional integrity, in the presence of excessive calcium load.

2. Materials and methods

2.1. Cell culture conditions

SK-N-SH neuroblastoma cells were used for the experiments of Figs. 1 and 2 obtained from American Type Tissue Collection (Rockville, MD). Cells were grown to confluency in RPMI-1640 media (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Sigma Chemical Co, St Louis, MO or Hyclone,

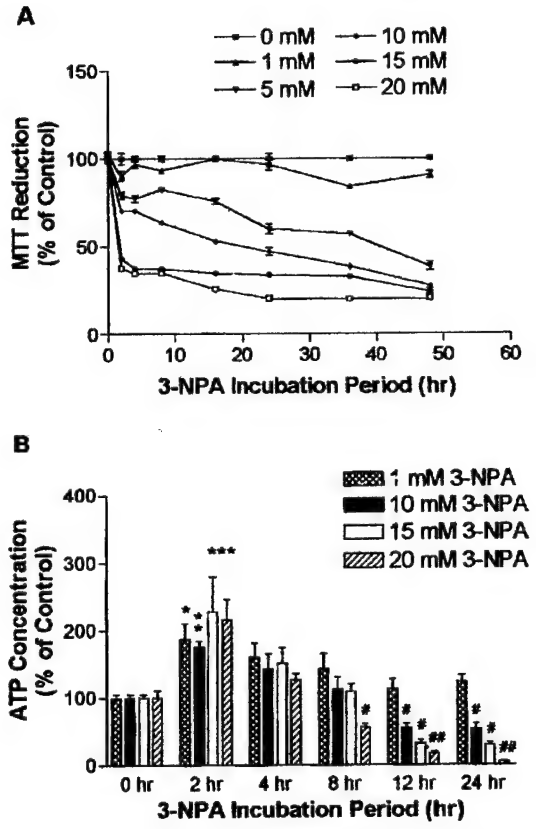

Fig. 1. Effect of 3-nitroproprionic acid (3-NPA) on MTT reduction and cellular ATP levels. (A) The time course of reduction of SDH by uncoupler 3-NPA at various doses was assessed in SK-N-SH cells. Values are expressed as a percentage of control levels and represent the mean ± SEM of determinations made in 8 cultures/group. The control values were the values in untreated cultures and were 0.863 ± 0.053 absorbance units per culture. (B) The effect of exposure to various doses of 3-NPA on levels in SK-N-SH cells was assessed. Values are expressed as a percentage of control levels and represent the mean ± SEM of determinations made in 3–6 cultures/group. Basal ATP levels in control cultures were 32.6 ± 7 nmol/mg protein. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; $\#p < 0.05$, $\#\#p < 0.01$ compared to 0 h groups (Wang et al., 2001).

Logan, UT) and 20 μg/ml gentamycin in monolayers in plastic Nunc 75 cm^2 flasks at 37 °C under 5% CO_2/95% air. Medium was changed three times weekly. Cells were observed with a phase-contrast microscope (Nikon Diaphot-300). SK-N-SH cells were back-cultured every 5–7 days using standard trypsinization procedures to maintain the cell line. SK-N-SH cells were used in passages 38–48. 3-NPA (Sigma) was initially dissolved at 100 mM then further diluted to the final concentration in experimental media as indicated.

Neuroblastoma SHSY-5Y cells, also obtained from American Type Tissue Collection, were used in the experiment shown in Fig. 3. Cells were propagated in high glucose DMEM. Twenty-four hours prior to assay, cells were trypsinized and plated in clear-bottom, black-walled, 96-well plates (Costar). Cells were plated at 60,000/well.

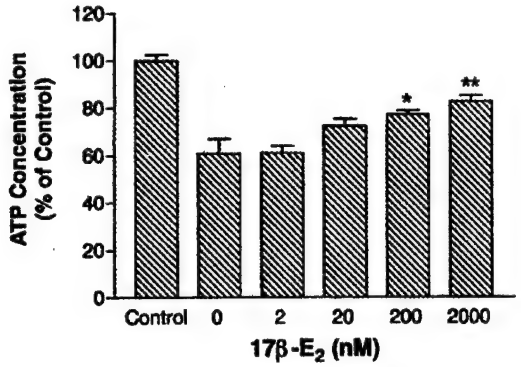

Fig. 2. 17β-E2 pretreatment prevents the decline in ATP levels in 3-NPA treated SK-N-SH cells. Cells were pretreated with 17β-E2 at various concentrations as indicated for 6 h and then exposed to 10 mM 3-NPA for 12 additional hours. ATP levels were determined as described in Section 2. Pretreatment with 17β-E2 in a dose-dependent manner prevented the decline in ATP levels following exposure to 3-NPA. Values are expressed as a percentage of basal levels and represent the mea ± SEM of determinations made for 4–8 cultures/group. Basal ATP levels in control cultures were 46.8 ± 7 nmol/mg protein. $*p < 0.05$, $**p < 0.01$ compared to the 0 nM 17β-E2 group (Wang et al., 2001).

2.2. Measurement of MTT reduction

The effects of 3-NPA on mitochondrial succinate dehydrogenase (SDH) activity were assessed indirectly by measuring the conversion of the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan crystals. Cells were plated at 2×10^4 cells per well in Nunc 96-well plates, and allowed to grow in regular media for 48 h. Cell cultures were exposed to various doses of 3-NPA various times between 2 and 48 h and were then rinsed with media and incubated with 10 μl MTT. Following overnight solubilization of the formazan crystals in 50% *N,N*-dimethylformamide, 20% sodium dodecyl sulfate, pH 4.8, the optical density was determined at 575/690 nm.

2.3. Measurement of ATP levels

Experiments were initiated by plating 1×10^6 cells per well in 24-well plates, and allowing growth in regular media for 48 h. Cells were exposed to (1) various doses of 3-NPA for 2–24 h, or (2) 10 mM 3-NPA for 2 or 12 h with or without various doses of 17β-E2 (6 h pretreatment, continued through 2 or 12 h, respectively). Cellular ATP levels were quantified using a luciferin and luciferase-based assay. Cells were rinsed with PBS and lysed with ATP-releasing buffer containing 100 mM potassium phosphate buffer at pH 7.8, 2 mM EDTA, 1 mM dithiothreitol (DTT) and 1% Triton X-100; 10 μl of the lysate was taken for protein determination. Another 10 μl of the lysate was taken and added to a Nunc 96-well plate. ATP concentrations in lysates were quantified using ATP determination kit (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. The 96-well plates were read using

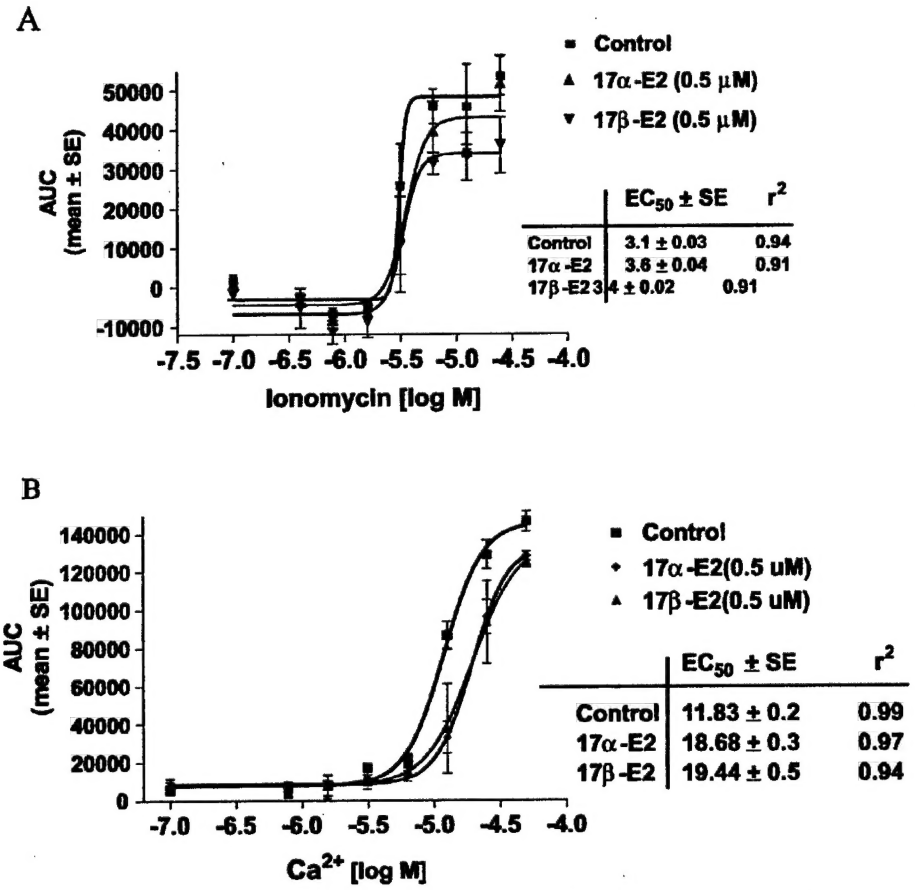

Fig. 3. 17α- and 17β-estradiol stabilize mitochondrial membrane potential ($\Delta\psi_m$) in SHSY-5Y neuroblastoma exposed to high levels of Ca^{2+}.$\Delta\psi_m$ was monitored using a fluorescence resonance energy transfer (FRET) assay (Dykens and Stout, 2001) in intact cells after acute exposure to ionomycin (panel A), or to Ca^{2+} in 4 and 5 respectively in permeabilized cells (panel B). Cells were pre-incubated with estradiols at 0.5 μM for 5min or 2.5 h prior to Ca^{2+} challenge, which was imposed by adding ionomycin (panel A) or Ca^{2+} directly to cells that had been permeabilized with digitonin (0.008% final concentration, 5 min) (panel B). The response, calculated as area under the curve, was plotted versus log Ca^{2+} concentration using sigmoid regression analysis.

a MLX Microtiter Plate Luminometer (Dynex Technologies Inc., Chantilly, VA). A standard curve was generated using solutions of known ATP concentrations. ATP levels were calculated as nM of ATP per mg of protein and normalized to levels in untreated control cultures.

2.4. Fluorescence resonance energy transfer assay for mitochondrial membrane potential

In this assay, cardiolipin in the inner mitochondrial membrane is stained using nonyl acridine orange (NAO). Because it photobleaches under ambient illumination, NAO solution was made fresh every 4 h by diluting a frozen stock solution (5.16 mM in ETOH, stored in dark) to 516 nM in warm HBSS. 20 μl dilute NAO was added directly to cells in 100 μl culture media (final concentration 86 nM). Cells were incubated with NAO for 6 min, after which media were removed and the cells washed 3 × with warm HBSS before being transferred to the plate reader where TMR was added (final concentration 150 nM). TMR was made fresh daily by diluting a frozen stock (40 mM in DMSO) into warm HBSS (or KCl media detailed below) to a concentration of 75 nM. All dyes were obtained from Molecular Probes (Eugene, OR). The laser on the Fluorescence Imaging Plate Reader (FLIPR; Molecular Devices, Sunnyvale, CA) was run at 600 mW. In order to focus on the quenching of NAO, while minimizing signal from TMR, the standard emission filter in FLIPR was replaced with a narrow bandpass filter of 525 ± 6 nm, obtained from Omega Optical (Burlington, VT).

When assessing $\Delta\psi_m$ in intact cells, acute Ca^{2+} loading and consequent mitochondrial collapse was induced via ionomycin exposure (Dykens and Stout, 2001). In a variation of the assay (Fig. 3(B)), the cells were washed with warm 125 mM KCl containing 1 mM $MgCl_2$, 4 mM each of succinate, glutamate and malate as oxidizible substrates, 2 mM HEPES, and 1 mM KH_2PO_4 pH 7.0, and were then permeabilized upon addition of TMR, to the same final concentration as above, diluted in KCl media containing 0.032% digitonin (final concentration 0.008%) in the FLIPR. When permeabilized, PT is induced by adding Ca^{2+} directly, rather than ionophores (Dykens and Stout, 2001).

Permeabilization with digitonin is used to increase bioavailability of the compounds at the mitochondrion.

3. Results

Incubation of SK-N-SH cells with 3-NPA led to a time- and dose-dependent decline in MTT (as measured by the inhibition of reduction of MTT to formazan crystals; Fig. 1(A)). When measured at 24 h, the 10 mM concentration of 3-NPA caused an approximate 50% reduction in MTT. These results confirm that 3-NPA is acting to reduce levels of SDH in these cells.

Exposure to 3-NPA at concentrations ranging from 1 to 20 mM resulted in an initial increase in ATP levels with peak levels occurring at 2 h followed by a time-related decrease thereafter (Fig. 1(B)). In control cultures, cellular ATP was 27.8 ± 7.0 nmol/mg protein ($n = 6$). ATP levels in cells treated with 1 mM 3-NPA decreased back to control levels by 12 h and continued to decrease through 24 h (Fig. 1(B)). Exposure to 10, 15 or 20 mM concentrations of 3-NPA led to declines in ATP of 40, 60, and 80%, respectively, compared to controls, by 12 h (Fig. 1(B)). ATP levels had further decreased by 24 h to 50, 70, and 95% of values in control cells. These results confirm that two markers of mitochondrial function, succinate dehydrogenase and ATP, are profoundly decreased in a time-dependent fashion when cells are incubated with the mitochondrial toxin, 3-NPA.

We next evaluated the effect of 17β-E2 on ATP levels in cells treated with 3-NPA. Treatment with 17β-E2 alone for 12 h exerted no significant effects on cellular ATP levels (data not shown). Six hours of pretreatment with 200 nM or greater of 17β-E2 prevented the profound decline in ATP concentrations which follow 12 h of exposure to 3-NPA (Fig. 2). This effect was dose-dependent because a greater protective effect was observed at 2000 nM than the effect noted at 200 nM. Estradiol at 2 or 20 nM did not prevent reduction in ATP levels. This study shows that incubation of cells with 200 or 2000 nM of 17β-E2 protects mitochondria from the 3-NPA induced decline in function as assessed by ATP levels.

We further examined mitochondrial responses to 17α- and 17β-estradiol using a proprietary assay for mitochondrial membrane potential which is based on fluorescence energy transfer (FRET) between two dyes that colocalize to the mitochondria. The FRET assay circumvents the confounding variables of plasma membrane potential and the low fluorescence efficiency typical of single potentiometric dyes (Dykens and Stout, 2001).

The excitation dye in the FRET $\Delta\psi_m$ assay is nonyl acridine orange (NAO). NAO is an exceptionally selective stain for cardiolipin, a lipid found intracellularly almost exclusively (>99%) in the mitochondrial inner membrane. Its staining of the inner membrane is independent of $\Delta\psi_m$. The second dye is tetramethylrhodamine (TMR), a potentiometric dye that is sequestered into the mitochondrial matrix as a Nernstian function of $\Delta\psi_m$. It is the specificity of NAO staining for cardiolipin, combined with the absolute prerequisite for close proximity of both dyes, that allows this FRET assay to report $\Delta\psi_m$ without interference from plasma membrane potential.

Because uptake and retention of TMR depends on $\Delta\psi_m$, at a given dye concentration, the extent of quenching of NAO by TMR reflects the magnitude of $\Delta\psi_m$. Dissipation of $\Delta\psi_m$ in response to elevated Ca^{2+} load or respiratory uncouplers, results in efflux of TMR from the mitochondrion. The corresponding loss of proximity abolishes quenching of NAO, so that loss of $\Delta\psi_m$ is detected as an increase in NAO fluorescence.

Results from the FRET assay show that 17α-estradiol as well as 17β-estradiol preserve mitochondrial membrane potential during excessive Ca^{2+} loading (Fig. 3(A) and (B)). In both intact (Fig. 3(A)) and digitonin-permeabilized neuroblastoma cells, 17α-estradiol and 17β-estradiol substantially increased the amount of Ca^{2+} required to induce $\Delta\psi_m$ dissipation. In intact cells, 17β-estradiol, but not 17α-estradiol, reduces the magnitude of $\Delta\psi_m$ reduction, as shown by persistant quenching of NAO even at the highest ionomycin concentrations ($p < 0.01$, ANOVA) Fig. 3(A)). Large variance in the responses undermine ability to resolve beneficial effect of 17α-estradiol. This is not the case, however, for permeabilized cells, where the EC_{50} values for both 17α-estradiol (18.68 ± 0.3 SE, $N = 4$) and 17β-estradiol (19.44 ± 0.5 SE, $N = 4$) were significantly increased over controls (11.83 ± 0.2 SE, $N = 4$) ($p < 0.01$, covariance analysis). These experiments suggest that at a given Ca^{2+} load, a larger portion of the mitochondrial population in cells treated with estrogens retain $\Delta\psi_m$, in accord with data showing preservation of adenylate charge and moderation of cell death under these circumstances (Wang et al., 2001; present data).

4. Discussion

Post-menopausal estrogen replacement therapy is associated with several beneficial neurological outcomes including a reduction in incidence of Alzheimer's disease (Tang et al., 1996; Kawas et al., 1997), Parkinson's disease (Marder et al., 1998; Saunders-Pullman et al., 1999), and death from stroke in epidemiological studies (Finucane et al., 1993). Results are consistent with the activity of estrogens as neuroprotective agents. Indeed, numerous studies in cellular and animal models have established that estrogens act as potent neuroprotectants when challenged with any one of a various of toxic stressors.

As described in Section 1, work from our laboratories and those of others have shown that neuroprotection can be achieved without invoking activity at a classical transcription-mediated estrogen receptor. Many lines of converging

evidence support this conclusion. Significantly, compounds that fail to bind either of the ER receptors to any appreciable extent, such as *ent*-17β-E2 and others, exhibit robust neuroprotective activities in a wide variety of in vitro and in vivo models. Structure-activity relationships optimizing polycyclic phenols for neuroprotection diverge fundamentally from those that optimize for hormonal responsiveness. Because estrogens have multiple actions including activation of hormonally responsive tissues via the estrogen receptors, however, and because such receptors are localized in the brain among other tissues, it is plausible that ER-mediated mechanisms also have a role in neuroprotection under certain circumstances.

Our goals are to identify and develop novel compounds that retain the neuroprotective activities associated with this class of compounds, but that do not bind to classical estrogen receptors and induce a hormonal response in vivo. To that end, we have assessed the cellular mechanisms of action of 17β-E2, 17α-E2, and other compounds that retain the essential phenolic A ring, the minimal structure requirement for neuroprotection (Green et al., 1997b). In the studies reported here, we have shown that 17β-E2 prevents the depletion of ATP resulting from exposure to the mitochondrial toxin, 3-NPA. We also show that both compounds protect mitochondria against loss of membrane potential in response to excessive calcium load. This is unlikely a genomic action since 17α-E2, which has affinity for the estrogen receptors 2–3 orders of magnitude lower than 17β-E2, is equally as effective in this assay.

Although the specific molecular mechanism(s) by which cells are protected by these compounds is still under investigation, the body of evidence increasingly suggests that the polycyclic phenols exert their cytoprotective effects at least in part by stabilizing mitochondrial function, and by so doing, moderating apoptotic and/or necrotic cell death associated with mitochondrial dysfunction.

Estrogen and novel polycyclic phenols have potential for use in the prevention or amelioration of symptoms of chronic neurodegenerative diseases, like Alzheimer's and Parkinson's disease, as well as in more acute neuronal compromising conditions such as stroke and traumatic brain injury. We are currently synthesizing and analyzing novel compounds that are active by this mechanism for use in treatment of these and other significant neurodegenerative diseases and conditions.

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